Neuregulin-1 improves right ventricular function and attenuates experimental pulmonary arterial hypertension

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Aims
Pulmonary arterial hypertension (PAH) is a serious disease that affects both the pulmonary vasculature and the right ventricle (RV). Current treatment options are insufficient. The cardiac neuregulin (NRG)-1/ErbB system is deregulated during heart failure, and treatment with recombinant human NRG-1 (rhNRG-1) has been shown to be beneficial in animal models and in patients with left ventricular (LV) dysfunction. This study aimed to evaluate the effects of rhNRG-1 in RV function and pulmonary vasculature in monocrotaline (MCT)-induced PAH and RV hypertrophy (RVH).

Methods and results
Male wistar rats (7- to 8-weeks old, n = 78) were injected with MCT (60 mg/kg, s.c.) or saline and treated with rhNRG-1 (40 μg/kg/day) or vehicle for 1 week, starting 2 weeks after MCT administration. Another set of animals was submitted to pulmonary artery banding (PAB) or sham surgery, and followed the same protocol. MCT administration resulted in the development of PAH, pulmonary arterial and RV remodelling, and dysfunction, and increased RV markers of cardiac damage. Treatment with rhNRG-1 attenuated RVH, improved RV function, and decreased RV expression of disease markers. Moreover, rhNRG-1 decreased pulmonary vascular remodelling and attenuated MCT-induced endothelial dysfunction. The anti-remodelling effects of rhNRG-1 were confirmed in the PAB model, where rhNRG-1 treatment was able to attenuate PAB-induced RVH.

Conclusion
rhNRG-1 treatment attenuates pulmonary arterial and RV remodelling, and dysfunction in a rat model of MCT-induced PAH and has direct anti-remodelling effects on the pressure-overloaded RV.

Keywords
Pulmonary hypertension • Right ventricular function • Neuregulin • Endothelial dysfunction • Cardiac hypertrophy

1. Introduction
Pulmonary arterial hypertension (PAH) is a progressive disease characterized by pulmonary arterial remodelling, elevated pulmonary vascular resistance (PVR), increased right ventricular (RV) afterload, and RV failure.¹ RV adaptation to loading and RV function are main predictors of the outcome in PAH.²

Current treatment of PAH consists of prostanoids, endothelin-1 antagonists, and phosphodiesterase inhibitors. These therapeutic interventions target pulmonary vascular endothelial dysfunction and pulmonary arterial vasoconstriction. Despite some clinical successes with these therapies, PAH remains a severe disease.³ Thus, new therapies for PAH should protect against RV maladaptation and failure.⁴ The mechanisms of RV dysfunction in PAH are, however, complex and multifactorial. Most likely, these mechanisms go beyond a mechanical overload⁵ and may be more systemic.⁶

The NRG-1/ErbB system is critical for cardiac development and is activated at an early stage of compensated heart failure, in conditions...
of myocardial stress, and decreases with disease progression and decompensation.\(^7,8\) NRG-1 acts through transmembrane tyrosine kinase receptors of the ErbB family that dimerize upon binding of NRG-1 to ErbB3 or ErbB4, leading to phosphorylation and downstream signalling. NRG-1 is released from cardiac endothelial cells, whereas ErbB2 (co-receptor), ErbB3, and ErbB4 receptors are expressed in cardiomyocytes and cardiac fibroblasts.\(^9,10\)

Administration of NRG-1 ameliorates cardiac dysfunction and reduces the mortality in several models of left ventricular (LV) failure.\(^11\) Treatment with NRG-1 improves LV function in volume overload,\(^12\) doxorubicin-induced LV dysfunction,\(^13\) and in ischaemic\(^14\) and diabetic cardiomyopathy.\(^15\) These findings have led to clinical trials that showed efficacy and safety of NRG-1 in improving LV function in patients with heart failure.\(^16,17\)

Apart from its role in endothelium–cardiomyocyte cross-talk,\(^18\) NRG-1 also reduces neointimal hyperplasia following vascular injury and inhibits proliferation of vascular smooth muscle cells,\(^19\) having a protective role in both smooth muscle and endothelial cells.\(^20\) These observations are relevant, since neointima formation and smooth muscle cell proliferation in pulmonary vessels are a hallmark of PAH.\(^1\)

Based on the actual knowledge described above, we hypothesize that by treating monocrotaline (MCT)-induced PAH animals with exogenous NRG-1 we might protect not only lung vessels, but also the RV and thus attenuate MCT-induced PAH and improve RV and overall myocardial function. In the present study, we evaluated the functional and structural effects of the administration of recombinant human NRG-1 (rhNRG-1) on the heart and pulmonary vessels in MCT-induced PAH in rats. To distinguish cardiac-specific actions from its effects on the pulmonary vasculature, rhNRG-1 treatment was also studied in an experimental model of pressure overload by pulmonary artery banding (PAB), which results in RV loading without PAH.

2. Materials and methods

All the procedures in this work followed the recommendations of the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, Revised 2011), are certified by the Portuguese Veterinary Governmental Association, approved by the Portuguese Foundation for Science and Technology (PTDC/SAU-FCF/100442/2008) and approved by the faculty ethical committee (0420/000/000/2010). All animal handling was performed by trained researchers, certified with a Laboratory Animal Science course according to the Federation of European Laboratory Animal Science Associations. A detailed description of methods is presented in Supplementary material online.

2.1 Animal models and experimental design

Seven- to eight-week-old male Wistar rats (Charles River Laboratories) weighing 180–200 g were randomly assigned to receive a subcutaneous injection of 60 mg/kg MCT (Sigma-Aldrich) or an equal volume of vehicle. Two weeks (14 days) after administration, rats were assigned to receive 40 μg/kg rhNRG-1 i.p. (Peprotech) or vehicle daily during 1 week, resulting in four groups: Ctrl + vehicle (Group C, n = 16); Ctrl + rhNRG-1 (Group CN, n = 14); MCT + vehicle (Group M, n = 24); MCT + rhNRG-1 (Group MN, n = 24). To determine whether MCT-induced PAH was already present prior to treatment, an additional group underwent the same experimental protocol and was evaluated at an earlier time point (14 days).

Another group of animals was submitted to PAB and to the same randomization, time points, and chronic treatment protocol (see Supplementary material online, Methods), resulting in four groups: Sham + vehicle (Group S, n = 8); Sham + rhNRG-1 (Group SN, n = 7); PAB + vehicle (Group B, n = 8); PAB + rhNRG-1 (Group BN, n = 10). Applying a 1.65 mm pulmonary artery constriction resulted in a degree of hypertrophy and RV overload identical to the MCT-induced PAH model (see Supplementary material online, Figure S1).

Three weeks (21 days) after MCT and PAB, rats were submitted to echocardiographic (MCT protocol) and haemodynamic evaluation, with subsequent sample collection for in vitro functional studies, morphological, histological, and molecular analysis.

2.2 Echocardiography

Rats were anaesthetized with an i.p. injection of ketamine/xylazine (75 and 10 mg/kg, respectively). Echocardiographic evaluation was performed using a 12 MHz probe (GE Healthcare) and a General Electrics Vivid 7 echocardiograph (GE Healthcare). The echocardiographic parameters assessed included PA acceleration and ejection time (PAAT and PAET, respectively), PA velocity-time integral (PAVTI), RV diastolic dimension (RVDD), right atrium area (RAA), and interventricular septum diastolic dimension (IVSD).

2.3 Invasive haemodynamic assessment

As previously described,\(^21\) rats were sedated (100 μg/kg and 5 mg/kg i.p., fentanyl and midazolam, respectively) and anaesthetized (inhalation of 8% sevofluorane for induction and 2–3.5% for maintenance) and intubated. Using an open chest approach, pressure–volume catheters were introduced in the RV and LV (SPR-869 and SPR-847, respectively, Millar Instruments). A flow probe was implanted around the ascending aorta (MA2.5PSB, Transonic Systems). Baseline and inferior vena cava occlusion recordings were obtained with ventilation suspended at end-expiration. Pressure and volume signals were continuously acquired (MPVS Ultra, Millar Instruments), digitally recorded (PowerLab 16/30, ADInstruments), and analysed off-line (LabChart 7 Pro, ADInstruments). Parallel conductance was computed after hypertonic saline bolus.

2.4 Sample collection and morphometric analysis

Following anaesthetic overdose, and immediately after exsanguination, heart and lungs were excised. RV free wall, LV + septum (LV + S), and lungs were dissected and weighed separately. Tibial length (TL) was used for normalization. RV samples were collected, snap frozen in liquid nitrogen, and stored at −80 °C. For mRNA quantification, samples were submerged in RNA stabilization reagent (RNAlater, Qiagen), and for histological analysis, samples were stored in buffered 10% formaldehyde.

2.5 Evaluation of RV and lung remodelling

After fixation, histological samples were embedded in paraffin, and sections were obtained from RV, lung, and isolated arterial rings. Haematoxylin and eosin (HE) staining was used to quantify cardiomyocyte and pulmonary artery morphology; Picro Sirius Red staining was used to quantify RV fibrosis; and Verhoeff–Van Gieson staining was used to measure isolated arterial rings remodelling. Sections were digitally photographed (Olympus XC30, Olympus) and measured using imaging software (Cell®B, Olympus). Pulmonary artery medial wall thickness was expressed as follows: %WT = ([Medial wall thickness × 2]/Arterial external diameter) × 100.

2.6 Assessment of isolated pulmonary artery endothelial function

Second-generation pulmonary arteries (200–400 μm diameter) were dissected from the left upper lobe of rats. Arterial rings were isolated and mounted in a bath myograph system (722OMO, DMT). Maximum tension development was assessed with 80 mM KCl solution, and a dose–response curve to acetylcholine was attained (10⁻²–10⁻¹ M, in 0.5 logarithmic units intervals), after pre-contraction with phenylephrine (10⁻³ M). At the end of the protocol, the arterial rings were collected for histological evaluation...
of pulmonary arterial remodelling of large diameter vessels. Maximal relaxation to acetylcholine (Emax) and the concentration of acetylcholine required for 50% of the maximal response (EC50) were calculated.

2.7 Quantitative RT-PCR, immunoblot, and cytokine ELISA

RV mRNA expression of NRG-1, B-type natriuretic peptide (BNP), caspase-3, endothelin-1 (ET-1), hypoxia inducible factor 1 alpha subunit (HIF-1α), IL-6, and TNF-α was quantified (primer sequences in Supplementary material online, Table S1). Lung mRNA expression of NRG-1, IL-6 and TNF-α was also quantified. Total mRNA was extracted using the RNeasy kit according to manufacturer’s instructions (Qiagen). Two-step RT-PCR was used for relative mRNA quantification (Step-One™, Applied Biosystems).

Blood was collected and centrifuged in EDTA-containing tubes for plasmatic quantification of IL-6 and TNF-α concentrations, using solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) according to manufacturer’s instructions (Rat IL-6 ELISA Kit and Rat TNF-α ELISA Kit, Invitrogen).

To determine rhNRG-1 activity, RV total protein was extracted from acutely treated animals and separated in a 10% SDS–PAGE gel, electroblotted into nitrocellulose membrane, and probed for ErbB4 according to manufacturer’s instructions (Rat IL-6 ELISA Kit and Rat TNF-α ELISA Kit, Invitrogen).

3. Results

3.1 rhNRG-1 improves pulmonary arterial flow and attenuates cardiac and pulmonary arterial remodelling in MCT-induced PAH

MCT-induced PAH results in a decrease in PA AT and PAVTI with a mid-systolic decrease in PA flow, RV dilation, and IVS hypertrophy and flattening. Accordingly, MCT animals presented altered PA flow, with faster acceleration and a consequent decrease in the PAAT/PAET ratio (Figure 1A), a mid-systolic notch (Figure 1F, white arrowhead) and decreased PAVTI (Figure 1B), representative of decreased stroke volume. Treatment with rhNRG-1 was able to normalize these changes, restoring pulmonary circulation. M group also presented RV dilation (Figure 1C), as measured by the dimension of the tricuspid annulus (Figure 1F), RA enlargement (Figure 1D), and IVS thickening (Figure 1E) and flattening, as shown by the rectilinear position of the IVS (Figure 1F). The aforementioned pathological heart remodelling (RV and RA increase and IVS thickening), observed in MCT animals through echocardiography, was restored by rhNRG-1 treatment.

Treatment with rhNRG-1 was able to attenuate body weight loss in MCT-treated animals (Figure 2A). In addition, the RV/LV + S ratio, a surrogate of RV hypertrophy, was greatly increased in the PAH group (Figure 2B) and was significantly attenuated by rhNRG-1 treatment. Together with this finding, pulmonary oedema, as quantified by the Lung/TL ratio, was also reduced by rhNRG-1 treatment compared with the MCT group (Figure 2C). This shows a decrease of fluid build-up in the lungs, potentially as a result of improved cardiac function and cardiac output (CO) in treated animals.

Animals with PAH and without pharmacological intervention presented increased cardiomyocyte cross-sectional area (CSA), as well as fibrosis deposition (Figure 2D and E). RhNRG-1 treatment normalized both cardiomyocyte size and fibrotic tissue deposition. Pulmonary small artery remodelling, measured by medial layer thickness, was also attenuated by rhNRG-1 treatment (Figure 2F).

3.2 rhNRG-1 amends reduced cardiac function in MCT-induced PAH

MCT-induced PAH resulted in RV dysfunction 3 weeks after MCT administration (Figure 3A). MCT animals also presented an increase in PVR (Figure 3B), and this resulted in higher right ventricular end-systolic pressure (ESP, Figure 3C), consistent with increased RV hypertrophy, RV dilation (increased end-diastolic volume, EDV, Figure 3D), and RV dysfunction as shown by the decrease of ejection fraction (EF, Figure 3E) and CO (Figure 3F), despite intrinsic myocardial contractility increase (higher load-independent contractility index, end-systolic elastance, Ees, Figure 3I). As mentioned above, by reducing pulmonary vascular remodelling, chronic treatment with rhNRG-1 was able to attenuate PVR, therefore reducing RV afterload and improving RV function. Consistently with increased fibrosis, MCT animals had diastolic dysfunction, with higher filling pressures, impaired relaxation, and increased diastolic stiffness, quantified by higher end-diastolic pressure (EDP, Figure 3G), increased isovolumic relaxation time constant (tau, Figure 3H), and increased end-diastolic elastance (Eed, Figure 3J), respectively. PAH animals treated with rhNRG-1 showed improved RV diastolic function, with a more compliant chamber, and restored relaxation, as shown by normalized Eed, EDP, and tau. Overall, chronic treatment with rhNRG-1 starting 2 weeks after MCT administration, when signs of PAH are already present (see Supplementary material online, Figure S2), was able to noticeably improve RV function 3 weeks after MCT administration.

Pressure–volume analysis of the LV (Figure 4A) showed decreased contractile LV performance in MCT-treated animals as shown by the decrease in ESP (Figure 4B), and was paralleled with decreased EDV (Figure 4C), and diastolic impairment (increased tau and Eed, Figure 4D and E, respectively). This might result from LV unloading subsequent to decreased RV ejection and septal bulging (shown by echocardiography). Similar to the RV, rhNRG-1 treatment improved global LV function, recovering both systolic and diastolic function.

3.3 rhNRG-1 attenuates pulmonary endothelial dysfunction in MCT-induced PAH

We found a lack of relaxation in a dose–response test to acetylcholine (Figure 5A and B) in pulmonary arteries isolated from MCT animals. Treating animals with rhNRG-1 did not change phenylephrine-induced maximal tension, but significantly enhanced endothelial function, by increasing the maximal response to acetylcholine by 12% (Figure 5C). Furthermore, rhNRG-1 decreased the EC50 (Figure 5D), increasing receptor sensitivity to acetylcholine. Pulmonary arterial remodelling
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was also reversed in arterial rings (large diameter vessels, Figure 5E and F) isolated from rhNRG-1-treated animals, in conformity with its effects on small diameter arteries (Figure 2F and G).

Besides decreasing pulmonary arterial remodelling, rhNRG-1 treatment improved endothelial function, contributing to the improvement of PVR in the treated group.

**Figure 1** rhNRG-1 improves pulmonary flow and RV structural changes in MCT-induced PAH. (A) Pulmonary acceleration time normalized to pulmonary ejection time (PAAT/PAET) is decreased in MCT animals and recovered with rhNRG-1 treatment. (B) Pulmonary artery velocity-time integral (PAVTI) is also decreased in PAH and restored with treatment. (C) RV end-diastolic diameter (RVEDD) is increased in MCT animals, while MCT animals treated with rhNRG-1 show no differences from control animals. (D) Right atria area (RAA) is higher in MCT animals and normalized in MCT animals treated with rhNRG-1. (E) Interventricular septum (IVS) thickness is higher in MCT animals and is normalized by rhNRG-1 treatment. (F) Representative images of pulmonary flow, and parasternal long axis (apical four-chamber view) and parasternal short axis of the heart of the different experimental groups. White arrowhead points to mid-systolic notch, and white arrows delineate the tricuspid valve (apical four-chamber view) and the IVS (short-axis view). Bars represent mean ± SEM of 8–11 rats per group. *P < 0.05 vs. Control; **P < 0.01 vs. Control; ***P < 0.05 vs. MCT. Two-way ANOVA was used for all the parameters presented.
Figure 2 MCT-induced RV and lung remodelling is attenuated by rhNRG-1 treatment. (A) Weight loss was evident in MCT animals, while treated animals showed a significantly higher body weight (BW). (B) RV hypertrophy, as measured by RV/LV + S ratio, was increased in MCT animals, while the RV of treated animals was significantly less hypertrophied. (C) Lung oedema, as measured by the Lung/TL ratio, was present in both MCT groups, and treatment with rhNRG-1 was able to attenuate this change. (D) RV cardiomyocyte CSA was increased in MCT-induced PAH, while rhNRG-1 treatment reversed cardiomyocyte hypertrophy. (E) RV fibrosis was also increased in MCT-induced PAH, and treatment with rhNRG-1 was able to normalize RV fibrosis. (F) Pulmonary arterial remodelling was increased in MCT animals, as shown by an increase in medial wall thickness, and was attenuated with rhNRG-1 treatment. (G) Representative photomicrographs of haematoxylin–eosin (HE) and Red Sirius staining of RV sections, and HE lung sections. Black scale lines represent 20 μm (×400 magnification) and 20 μm (×200 magnification) and 100 μm (×200 magnification) for RV HE and Red Sirius, and lung HE photomicrographs, respectively. Bars represent mean ± SEM of 14–16 rats per control group and 24 rats per MCT group for the morphometric data, and 6–12 rats per group in the histological data. *P < 0.05 vs. Control; ***P < 0.001 vs. Control; #P < 0.05 vs. MCT; ##P < 0.01 vs. MCT; ###P < 0.001 vs. MCT. Two-way ANOVA was used for all the parameters presented.
3.4 rhNRG-1 abrogates molecular changes in the RV and attenuates systemic inflammation in MCT-induced PAH

MCT-induced PAH resulted in an increase in NRG-1 gene expression in the RV (Figure 6A), which is associated with impaired RV function, as observed by a negative significant correlation between NRG-1 and EF (Figure 6B). No changes were observed in NRG-1 expression in the lung of the different experimental groups (Figure 6C). Animals treated with rhNRG-1 showed a reversal of RV NRG-1 expression to control levels compared with the MCT group without treatment. As expected, administration of rhNRG-1 resulted in ErbB4 receptor phosphorylation (Figure 6D), demonstrating the binding of the peptide to the receptor and its activation.

MCT animals presented increased RV expression of markers of hypertrophy and overload, namely, ET-1 (Figure 6E) and BNP (Figure 6F). We also found increased RV expression of caspase-3, as a surrogate for apoptosis (Figure 6G), and of HIF-1α as a tissue hypoxia marker (Figure 6H). RhNRG-1 treatment was able to restore the RV expression levels of all the mentioned cardiac damage markers.

Although myocarditis has been reported as a ‘side effect’ of MCT administration,23 we did not find changed RV pro-inflammatory cytokine expression (Figure 6I and J). We did find increased IL-6 (Figure 6L) expression in the lung of both MCT groups, demonstrating that pulmonary inflammation, secondary to MCT administration, was not attenuated by rhNRG-1.

3.5 rhNRG-1 improves RV structure in animals with RV hypertrophy induced by PAB

Using the PAB model, we sought to distinguish rhNRG-1’s effect on the RV, independent from its effect on the pulmonary vasculature. PAB surgery resulted in compensated RV hypertrophy, as measured by the RV/LVS ratio (Figure 7A), increased cardiomyocyte CSA (Figure 7B) and fibrosis (Figure 7C), and preservation of RV function, as seen by an unchanged CO (Figure 7D). RhNRG-1 treatment attenuated RV structural changes, by decreasing RV hypertrophy and fibrosis...
4. Discussion

In this work, we tested the effect of rhNRG-1 treatment in a rat model of MCT-induced PAH and RV overload. Consistent with our hypothesis, rhNRG-1 attenuated the severity of this disease, as evident from the salutary effects of rhNRG-1 on pulmonary and RV remodelling and overall cardiac function. Beneficial effects of rhNRG-1 were evident both at the functional and at the histological/structural level. Furthermore, using a model of pressure loading of the RV without PAH, we also demonstrated that rhNRG-1 treatment has direct beneficial effects on RV structure, by reducing hypertrophy and fibrosis.

The cardiac NRG-1/ErbB system has been intensely studied, and there is compelling evidence that this system is activated during compensated LV failure. Treatment of various animal models with LV dysfunction has resulted in improved cardiac function, LV remodelling, and reduced heart failure mortality, and has instigated ongoing clinical trials with NRG-1 in heart failure. Although it is generally believed that beneficial effects of NRG-1 in heart failure mainly result from direct effects on cardiomyocytes and, perhaps, on cardiac fibroblasts, the physiological effects of NRG-1 may be more pleiotropic, including effects on vascular endothelial cells, vascular smooth muscle cells, and inflammatory cells.

In line with these observations, the favourable effects of NRG-1 observed in the present study seem to result from effects on both the pulmonary vasculature (MCT model) and directly on the RV myocardium (PAB model). Both pulmonary arterial medial hypertrophy and pulmonary arterial endothelial dysfunction were markedly attenuated by NRG-1. This led to reduction of pulmonary arterial resistance, RV afterload, and consequently of RV hypertrophy and RV contractility. Although the precise mechanisms of these beneficial effects of NRG-1 on the pulmonary endothelium and vasculature remain to be deciphered, inhibitory effects of rhNRG-1 on PDGF-induced smooth muscle cell proliferation and stimulatory actions on nitric oxide synthesis may participate.

Lung inflammation is associated with the development of PAH, and inflammatory markers such as TNF-α and IL-6 are increased in MCT-induced PAH. Our finding that rhNRG-1 treatment did not attenuate lung inflammation shows that the improvement of pulmonary and RV function and structure was not achieved through attenuation of an acute inflammatory response in MCT-induced PAH. In the same perspective and regardless of previous evidence associating inflammatory cardiomyopathy to MCT-induced PAH, we did not observe TNF-α and IL-6 altered expression in the RV of MCT animals, which suggests that RV myocardial inflammation does not seem to play a role in our experimental setting. Despite this observation, PAH-associated systemic inflammation was attenuated by rhNRG-1 treatment, possibly as a result of overall improved function, revealing NRG-1’s potential function as an anti-inflammatory agent in PAH. Also, control animals treated with rhNRG-1 did not show increased pro-inflammatory cytokine levels showing that intraperitoneal administration of this peptide does not elicit an inflammatory response by itself.
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Besides decreasing vascular remodelling and dysfunction, NRG-1 seems to also act on the myocardium in MCT-induced PAH. The anti-hypertrophic effects of NRG-1 in the RV observed in this study are consistent with previous observations in which rhNRG-1 inhibits LV cardiomyocyte hypertrophy during post-infarct remodelling. Strikingly, in the MCT-induced PAH model, rhNRG-1 also prevented LV dysfunction. LV contractile dysfunction and impaired relaxation are generally present in PAH and were both attenuated by rhNRG-1 treatment. LVEDV, which was restored with treatment, was lower in rats with PAH. In PAH patients, these LV functional parameters are associated with increased mortality, underscoring the putative translational implication of this NRG-1 effect.

In PAB, a model without vascular disease, but with an identical degree of RV overload and hypertrophy to the MCT-induced PAH model used, rhNRG-1 was also able to mitigate hypertrophy and fibrosis, demonstrating that in fact a direct effect on the RV myocardium is present and importantly contributes to the improved RV function and structure observed in MCT animals treated with rhNRG-1.

Myocardial remodelling, increased wall stress, hypoxic damage, and apoptosis are associated with MCT-induced PAH increased RV expression of ET-1, BNP, HIF-1α, and caspase-3. Accordingly, all these markers were up-regulated in the RV of MCT animals, agreeing with the functional and structural changes observed. Either by directly acting on these signalling pathways, potentially regulating its expression, or by decreasing RV remodelling and improving its function, rhNRG-1 treatment was able to restore the expression of all the above-mentioned RV damage markers. In the present study, we also observed that NRG-1 was endogenously up-regulated during PAH. RV NRG-1 mRNA expression was increased in MCT animals and was associated with poorer RV function, possibly as a result of increased afterload and myocardial stress.

The beneficial effects of NRG-1 on both heart and vessels, by acting on cardiomyocytes, cardiac fibroblasts, endothelial cells, vascular smooth muscle cells, and inflammatory cells, might be an advantage in the treatment of PAH, compared with current therapeutic agents that are more focused on arterial pulmonary vasoconstriction. Clinical translation of these observations is ongoing, especially with regard to the treatment of heart failure.
Previous studies have shown that 2 weeks after MCT administration, rats already present increased RV and pulmonary dysfunction and remodelling. Our data (see Supplementary material online, Figure S2) show that, 14 days after MCT administration, animals develop RV hypertrophy with maintained function, lung oedema, possibly as a result of an early inflammatory response, and compromised pulmonary flow, where PAVTI and PAAT/PAET are already as decreased as 21 days after MCT administration (data not shown). This confirms that 2 weeks after MCT administration pulmonary dysfunction is established.

This finding suggests that treatment with rhNRG-1 recovers already established pulmonary flow dysfunction, attenuating overload of the RV and improving its function and structure. Therefore, by beginning rhNRG-1 treatment at day 14, we showed that rhNRG-1 has a role in treating already established PAH, thus facilitating its transition to clinical practice.

Limitations of our work include the lack of subcellular mechanisms for the beneficial role of the NRG-1, and although potential mechanisms were suggested, this will be the object of another line of research.
Additionally, the plexiform lesions that are found in the lungs of PAH patients, as well as in angioproliferative models of PH, are not usually seen in the MCT model. Still, the MCT model shares several main characteristics with both primary and secondary pulmonary hypertension in humans, such as pulmonary vascular remodelling, as well as RV and endothelial dysfunction.32 As rhNRG-1 ameliorates most of these parameters, we propose that NRG-1 could potentially serve as a treatment option for both forms of pulmonary hypertension in humans.

In conclusion, this study shows, for the first time, that NRG-1/ErbB signalling may have an important role in PAH and RV dysfunction and that rhNRG-1 treatment improves both cardiopulmonary function and structure. NRG-1 decreases pulmonary arteries remodelling, improves endothelial function, and restores RV function. These beneficial effects may improve outcome in PAH. These data should encourage further studies to elucidate the underlying mechanisms through which NRG-1 attenuates the pathophysiology of PAH.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Conflict of interest:** none declared.

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