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

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ABSTRACT

Aims: Pulmonary arterial hypertension (PAH) is a serious disease that affects both the pulmonary vasculature and the right ventricle (RV). Current treatments 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 ventricle (LV) dysfunction. This study aimed to evaluate the effects of rhNRG-1 in RV function and pulmonary vasculature in monocrotaline-induced PAH and RV hypertrophy (RVH).

Methods and Results: Male wistar rats (7-8 week old, n=78) were injected with monocrotaline (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, were 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 monocrotaline-induced PAH, and has direct anti-remodelling effects on the pressure-overloaded RV.

Key words: pulmonary hypertension, right ventricular function, neuregulin, endothelial dysfunction, cardiac hypertrophy
INTRODUCTION

Pulmonary arterial hypertension (PAH) is a progressive disease characterized by pulmonary arterial remodelling, elevated pulmonary vascular resistance, increased right ventricular (RV) afterload and RV failure. RV adaptation to loading and RV function are main predictors of outcome in PAH. 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. Thus, new therapies for PAH should protect against RV maladaptation and failure. The mechanisms of RV dysfunction in PAH are, however, complex and multifactorial. Most likely, these mechanisms go beyond a mechanical overload and may be more systemic.

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. 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.

Administration of NRG-1 ameliorates cardiac dysfunction and reduces the mortality in several models of left ventricular (LV) failure. Treatment with NRG-1 improves LV function in volume overload, doxorubicin-induced LV dysfunction, and in ischemic.
diabetic cardiomyopathy\textsuperscript{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\textsuperscript{16, 17}.

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

**MATERIALS AND METHODS**

All the procedures in this work followed the recommendations of the Guide for the Care and Use of Laboratory Animal, 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 Sciences course according to the Federation of European Laboratory Animal Science Associations. A detailed description of methods is presented in Supplementary material.

**Animal models and experimental design**

Seven-to-eight week-old male Wistar rats (Charles River Laboratories) weighing 180–200g, were randomly assigned to receive a subcutaneous injection of 60mg/kg monocrotaline (MCT, Sigma-Aldrich) or an equal volume of vehicle. Two weeks (14 days) after administration, rats were assigned to receive 40μg/kg rhNRG-1 i.p. (Peprotech) or vehicle daily during 1 week, resulting in 4 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). In order to determine if 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 pulmonary artery banding (PAB), and submitted to the same randomization, time points, and chronic treatment protocol (see supplementary methods), resulting in 4 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 Figure S1).

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

Rats were anesthetized with an i.p. injection of ketamine/xylazine (75mg/kg 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 (IVSDD).

Invasive hemodynamic assessment

As previously described\(^2\), rats were sedated (100 μg/kg and 5 mg/kg i.p., fentanyl and midazolam, respectively) and anesthetized (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 analyzed off-line (LabChart 7 Pro, ADInstruments). Parallel conductance was computed after hypertonic saline bolus.

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.

**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: 

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\%WT = \left(\frac{\text{Medial wall thickness} \times 2}{\text{Arterial external diameter}}\right) \times 100.
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**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 (720MO, DMT). Maximum tension development was assessed with 80 mM KCl solution, and a dose-response curve to acetylcholine was attained (10^{-9} to 10^{-5} M, in 0.5 logarithmic units intervals), after pre-contraction with phenylephrine (10^{-5} 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.
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), interleukin 6 (IL-6), (NRG-1) and tumor necrosis factor alpha (TNF-α) was quantified (primer sequences in Table S1). Lung mRNA expression of NRG-1 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). Results are expressed in arbitrary units (AU), normalized to GAPDH, which did not differ between groups.

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-Immuno-Sorbent Assay (ELISA) according to manufacturer’s instructions (Rat IL-6 ELISA Kit and Rat TNF-α ELISA Kit, Invitrogen).

In order to determine rhNRG-1 activity, RV total protein was extracted from acutely treated animals and separated in a 10% SDS-PAGE gel, electro-blotted into nitrocellulose membrane and probed for ErbB4 (ErbB4 (C-18): sc-283, SantaCruz Biotechnology) and phospho-ErbB4 (Phospho-HER4/ErbB4 (Tyr1284)(21A9) #4757, Cell Signaling Technologies).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc.). 2-way ANOVA was used to statically analyze all the presented parameters (which followed a normal distribution). Endothelial function was analysed with a 2-way repeated measures ANOVA test, and the comparison of control and MCT animals 14 days after MCT administration was analysed with the t-student. Holm-Sidak’s method was performed for post hoc comparisons between groups. Group data are presented as means ± SEM and differences with p<0.05 were considered statistically significant.
RESULTS

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 PAAT and PAVTI with a midsystolic decrease in PA flow, RV dilation, and IVS hypertrophy and flattening\(^{22}\). Accordingly, MCT animals presented altered PA flow, with faster acceleration and a consequent decrease in the PAAT/PAET ratio (Figure 1 – A), a midsystolic notch (Figure 1 – F, white arrowhead) and decreased PAVTI (Figure 1 – B), 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 1 – C), as measured by the dimension of the tricuspid annulus (Figure 1 – F), RA enlargement (Figure 1- D), IVS thickening (Figure 1 – E) and flattening, as showed by the rectilinear position of the IVS (Figure 1 – F). 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 2 – A). In addition, the RV/LV+\(S\) ratio, a surrogate of RV hypertrophy, was greatly increased in the PAH group (Figure 2 – B), 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 when compared to the MCT group (Figure 2 – C). 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, as well as fibrosis deposition (Figure 2 – D 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 2 – F).

**RhNRG-1 amends reduced cardiac function in MCT-induced PAH**

Monocrotaline-induced PAH results in RV dysfunction 3 weeks after MCT administration (Figure 3 – A). MCT animals also present an increase in pulmonary vascular resistance (PVR, Figure 3 – B) and this results in higher right ventricular end-systolic pressure (ESP, Figure 3 – C), consistent with increased RV hypertrophy, RV dilation (increased end-diastolic volume, EDV, Figure 3 – D), and RV dysfunction as shown by the decrease of ejection fraction (EF, Figure 3 – E) and CO (Figure 3 – F), despite intrinsic myocardial contractility increase (higher load-independent contractility index, end-systolic elastance, Ees, Figure 3 – I). As mentioned above, by reducing pulmonary vascular remodeling, 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 3 – G), increased isovolumic relaxation time constant (tau, Figure 3 – H) and increased end-diastolic elastance (Eed, Figure 3 – J), 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 (Figure S2), was able to noticeably improve RV function 3 weeks after MCT administration.

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

**RhNRG-1 attenuates pulmonary endothelial dysfunction in MCT-induced PAH**

We found a lack of relaxation in a dose-response test to acetylcholine (Figure 5 – A 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 5 – C). Furthermore, rhNRG-1 decreased the EC50 (Figure 5 – D), increasing receptor sensitivity to acetylcholine. Pulmonary arterial remodelling was also reversed in arterial rings (large diameter vessels, Figure 5 – E and F) isolated from rhNRG-1 treated animals, in conformity with its effects on small diameter arteries (Figure 2 – F and G).

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

**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 6 – A), which is associated with impaired RV function, as observed by a negative significant correlation between NRG-1 and EF (Figure 6 – B). No changes were observed in NRG-1 expression in the lung of the different experimental groups (Figure 6 – C). Animals treated with rhNRG-1 showed a reversal of RV NRG-1 expression to control levels when compared
to the MCT group without treatment. As expected, administration of rhNRG-1 resulted in ErbB4 receptor phosphorylation (Figure 6 – D), 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 6 – E) and BNP (Figure 6 – F). We also found increased RV expression of caspase-3, as a surrogate for apoptosis (Figure 6 – G) and of HIF-1α as a tissue hypoxia marker (Figure 6 – H). 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, we did not find changed RV pro-inflammatory cytokine expression (Figure 6 – I and Figure 6 – J). We did find increased IL-6 (Figure 6 – L) expression in the lung of both MCT groups, demonstrating that pulmonary inflammation, secondary to MCT administration, was not attenuated by rhNRG-1.

However, plasmatic levels of TNF-α (Figure 6 – M) and IL-6 (Figure 6 – N), which were increased in animals from the MCT group, pointing to systemic inflammation, were attenuated by rhNRG-1 treatment.

**RhNRG-1 improves RV structure in animals with RV hypertrophy induced by pulmonary artery banding**

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 7 – A), increased cardiomyocyte CSA (Figure 7 – B) and fibrosis (Figure 7 – C), and preservation of RV function, as seen by an unchanged CO (Figure 7 – D). RhNRG-1 treatment attenuated RV structural changes, by decreasing RV hypertrophy and fibrosis in the PAB model, demonstrating that it has also
direct myocardial effects that are independent from its effects on pulmonary vasculature seen in MCT animals.

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 platelet-derived growth factor induced smooth muscle cell proliferation\textsuperscript{19}, and stimulatory actions on nitric oxide synthesis may participate\textsuperscript{26, 30}.

Lung inflammation is associated with the development of PAH\textsuperscript{31}, and inflammatory markers as TNF-\(\alpha\) and IL-6 are increased in MCT-induced PAH\textsuperscript{32}. 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\textsuperscript{23}. In the same perspective and regardless of previous evidence associating inflammatory cardiomyopathy to MCT-induced PAH\textsuperscript{23}, we did not observed TNF-\(\alpha\) 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\textsuperscript{33, 34}, 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 proinflammatory cytokine levels showing that intraperitoneal administration of this peptide does not elicit an inflammatory response by itself.

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\textsuperscript{25}. Strikingly, in the MCT-induced PAH model, rhNRG-1 also prevented LV dysfunction. LV contractile dysfunction\textsuperscript{35},\textsuperscript{36} and impaired relaxation\textsuperscript{37} 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\textsuperscript{38} these LV functional parameters are associated with increased mortality, underscoring the putative translational implication of this NRG-1 effect.

In the PAB model, 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\textsuperscript{39}, BNP\textsuperscript{40}, HIF-1α\textsuperscript{41} and caspase-3\textsuperscript{42}. Accordingly, all these markers were upregulated 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 upregulated 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\textsuperscript{8}.
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, when 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 two weeks after MCT administration, rats already present increased RV and pulmonary dysfunction and remodeling. Our data (Figure S2) shows 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. 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.

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**Conflict of interest**

No conflict of interest to declare
REFERENCES


Figure Legends

**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 4 chamber view) and parasternal short axis of the heart of the different experimental groups. White arrowhead points to midsystolic notch, and white arrows delineate the tricuspid valve (Apical 4 chamber view) and the IVS (short axis view). Bars represent mean ± SEM of 8-11 rats per group. *P<0.05 vs Control; ***P<0.001 vs Control; #P<0.05 vs MCT; ##P<0.01 vs MCT; ###P<0.01 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 cross sectional area (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 (H&E) and Red Sirius staining of RV sections, and H&E lung sections. Black scale lines represent 20 µm (400X magnification) and 20 µm (200X magnification) and 100 µm (200X magnification) for RV H&E and Red Sirius, and lung H&E 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<0.01 vs MCT. Two-way ANOVA was used for all the parameters presented.

Figure 3 – RhNRG-1 treatment improves RV function in MCT-induced PAH. (A) Representative pressure-volume loops of the different experimental groups. (B) Pulmonary vascular resistance (PVR) is increased in MCT animals and is attenuated with rhNRG-1 treatment. (C) MCT animals show higher RV end-systolic pressure (ESP), while MCT animals treated with rhNRG-1 show a significant reduction of ESP. (D) RV dilation, as measured by the end-diastolic volume (EDV) occurs in MCT-induced PAH, while treatment restores RV volume. (E) RV ejection fraction (EF) is compromised in MCT animals, and normalized in MCT animals treated with rhNRG-1. (F) Cardiac output (CO) is severely decreased in MCT-induced PAH and significantly improved with treatment. (H) Relaxation,
as measured by the isovolumic relaxation time constant (tau) is compromised (increased tau) in MCT animals and is normalized in MCT animals treated with rhNRG-1. (I) End-systolic elastance (Ees) is increased in MCT animals and significantly attenuated in MCT animals treated with rhNRG-1. (J) End-diastolic elastance (Eed) is increased in MCT-induced PAH and is restored by rhNRG-1 treatment. Bars represent mean ± SEM of 14-16 rats per control group and 24 rats per MCT group. *P<0.05 vs Control; **P<0.01 vs Control; ***P<0.001 vs Control; #P<0.05 vs MCT; ##P<0.01 vs MCT; ###P<0.01 vs MCT. Two-way ANOVA was used for all the parameters presented.

Figure 4 – RhNRG-1 treatment improves left ventricular function in MCT-induced PAH. (A) Representative pressure-volume loops of the different experimental groups. (B) LV end-systolic pressures were decreased in MCT animals, and treatment with rhNRG-1 reversed this change. (B) End-diastolic volume was decreased in MCT-induced PAH and normalized with rhNRG-1 treatment. (D) Isovolumic relaxation time constant (tau) was increased in MCT animals and normalized with rhNRG-1 treatment. (E) End-diastolic elastance (Eed) was increased in MCT-induced PAH and normalized with rhNRG-1 treatment. Bars represent mean ± SEM of 14-16 rats per control group and 24 rats per MCT group. **P<0.01 vs Control; ***P<0.001 vs Control; #P<0.05 vs MCT; ##P<0.01 vs MCT; ###P<0.01 vs MCT. Two-way ANOVA was used for all the parameters presented.

Figure 5 – Treatment with rhNRG-1 attenuates endothelial dysfunction and attenuates large diameter pulmonary arteries remodelling. (A) MCT-induced PAH resulted in decreased vasorelaxation induced by acetylcholine, while chronic treatment with rhNRG-1 improved vasorelaxation. (B) Representative tracings of the different acetylcholine dose-response curves. (C) Acetylcholine-induced relaxation maximal response (Emax) was decreased in
MCT animals, and significantly improved in MCT animals treated with rhNRG-1. (D) Dose-response curve to acetylcholine EC50 was increased in MCT-induced PAH, and attenuated by rhNRG-1 treatment. (E) Isolated large diameter pulmonary arteries wall thickness was increased in MCT animals and normalized in MCT animals treated with rhNRG-1. (F) Representative photomicrographs of isolated pulmonary arteries stained with Verhoeff–Van Gieson stain. Black scale line represents 200 µm (200X magnification). Bars represent mean ± SEM of 6-8 rats per group. *P<0.05 vs Control; **P<0.01 vs Control; ***P<0.001 vs Control; *P<0.05 vs MCT; **P<0.01 vs MCT. Two-way repeated measures ANOVA was used for the dose-response curve to acetylcholine, while two-way ANOVA was used for all the other parameters presented.

**Figure 6** – Expression of cardiac disease markers is reversed by rhNRG-1 treatment. (A) RV NRG-1 mRNA levels are increased in MCT-induced PAH and normalised with rhNRG-1 treatment. (B) Increased levels of RV NRG-1 are negatively correlated with EF (Pearson r = -0.8782; P<0.0001). Data used for this correlation analysis was obtained from animals that had both PV-Loop analysis and mRNA quantification of RV NRG-1. (C) Lung NRG-1 mRNA expression does not change in MCT-induced PAH. (D) Administration of rhNRG-1 results in ErbB4 receptor phosphorylation. ET-1 (E), BNP (F), Caspase-3 (G) and HIF-1α (H) upregulation is normalized by rhNRG-1 treatment. RV proinflammatory cytokine - TNF-α (I) and IL-6 (J) - expression is unchanged in all the experimental groups. Lung proinflammatory cytokine TNF-α (K) expression is not changed, while IL-6 (L) expression is increased in MCT-induced PAH and is not affected by rhNRG-1 treatment. Systemic inflammation, as measured by plasmatic levels of TNF-α (M) and IL-6 (N) is increased in MCT-induced PAH and attenuated by rhNRG-1 treatment. Bars represent mean ± SEM of 6-12 rats per group.
*P<0.05 vs Control; **P<0.01 vs Control; ***P<0.001 vs Control; #P<0.05 vs MCT; ##P<0.01 vs MCT. Two-way ANOVA was used for all the parameters presented.

**Figure 7** – PAB-induced RV hypertrophy and fibrosis are attenuated by rhNRG-1 treatment. Treatment with rhNRG-1 attenuated the (A) PAB increased RV/LV+S ratio (B) PAB induced cardiomyocyte hypertrophy, quantified as increased cardiomyocyte cross sectional area (CSA), (C) PAB increased fibrosis. (D) Cardiac output (CO) was similar in all experimental groups. (E) Representative photomicrographs of haematoxylin-eosin (H&E) and Red Sirius staining of RV sections. Black scale lines represent 20 µm (40X magnification) and 50 µm (25X magnification) for H&E and Red Sirius, respectively. Bars represent mean ± SEM of 6-8 rats per group. *P<0.05 vs Sham; **P<0.01 vs Sham; ***P<0.001 vs Sham; #P<0.05 vs Banding. Two-way ANOVA was used for all the parameters presented.