Neuregulin-1 attenuates right ventricular diastolic stiffness in experimental pulmonary hypertension

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Abstract

We have previously shown that treatment with recombinant human neuregulin-1 (rhNRG-1) improves pulmonary arterial hypertension (PAH) in a monocrotaline (MCT)-induced animal model, by decreasing pulmonary arterial remodeling and endothelial dysfunction, as well as by restoring RV function. Additionally, rhNRG-1 treatment showed direct myocardial anti-remodelling effects in a model of pressure loading of the RV without PAH. This work aimed to study the intrinsic cardiac effects of rhNRG-1 on experimental PAH and RV pressure overload, and more specifically on diastolic stiffness, at both the ventricular and cardiomyocyte level. We studied the effects of chronic rhNRG-1 treatment on ventricular passive stiffness in RV and LV samples from MCT-induced PAH animals and in the RV from animals with compensated and decompensated RV hypertrophy, through a mild and severe pulmonary artery banding (PAB). We also measured passive tension in isolated cardiomyocytes and quantified the expression of myocardial remodelling-associated genes and calcium handling proteins. Chronic rhNRG-1 treatment decreased passive tension development in RV and LV isolated from animals with MCT-induced PAH. This decrease was associated with increased phospholamban phosphorylation, and with attenuation of the expression of cardiac maladaptive remodelling markers. Finally, we showed that rhNRG-1 therapy decreased RV remodeling and cardiomyocyte passive tension development in PAB-induced RV hypertrophy animals, without compromising cardiac function, pointing to cardiac specific effects in both hypertrophy stages. In conclusion, we demonstrated that rhNRG-1 treatment decreased RV intrinsic diastolic stiffness, through the improvement of calcium handling and cardiac remodelling signalling.

Keywords: Diastolic Stiffness; Diastolic Function; Neuregulin-1; Pulmonary Arterial Hypertension; Right Ventricle.

1. Introduction

Patients with pulmonary arterial hypertension (PAH) develop heart failure (HF) due to a progressive increase in right ventricular (RV) pressure overload (1). Although it is known for some years that RV systolic adaptation is of clinical importance, it just recently became clear that RV diastolic stiffness increases and may contribute to disease progression in PAH (2, 3). Hypertrophy, fibrosis and stiffening of the RV cardiomyocytes all appeared to contribute to the observed RV diastolic stiffness. Indeed, there are stage-specific changes in the RV structure in PAH patients, which influence its diastolic function (4), and new PAH therapeutic strategies should also protect against RV maladaptation and failure (5).

Neuregulin-1 (NRG-1), a member of the epidermal growth factor (EGF) family, acts through tyrosine kinase receptors (human epidermal growth factor receptor 2, 3 and 4 - ErbB2, ErbB3, and ErbB4) that dimerize upon ligand binding, leading to phosphorylation and downstream signaling (6). The cardiac NRG-1/ErbB system is critical for cardiac development and is activated during compensated left ventricular (LV) failure (7). Additionally, a variety of studies in experimental LV dysfunction have shown favorable effects of NRG-1 treatment, resulting in improved cardiac function, LV remodelling, and reduced HF mortality (8-12), which were translated into clinical trials, showing efficacy and safety in patients with HF (13, 14). Additional studies have been completed (ClinicalTrials.gov Identifier: NCT01251406), and future studies aiming to determine the effects of rhNRG-1 on survival are ongoing (ClinicalTrials.gov Identifier: NCT03388593).

Recently, we have demonstrated that chronic recombinant human NRG-1 (rhNRG-1) therapy attenuated experimental monocrotaline (MCT)-induced PAH, as evidenced by beneficial effects on pulmonary and RV remodelling and function (15). Also, in a model of pressure loading of the RV without PAH (16), rhNRG-1treatment has direct beneficial effects on RV structure (15). However, the role of rhNRG-1 in cardiomyocyte stiffness, myofilament Ca^{2+} sensitivity and molecular changes that contribute to the impairment of RV diastolic function in PAH need to be clarified.

Therefore, this work aimed to study rhNRG-1's effects on isolated cardiomyocytes in experimental PAH and RV pressure overload, specifically on passive tension development. Furthermore, we aimed to determine underlying mechanisms for rhNRG-1 modulation of RV diastolic function.

2. Results

2.1 RhNRG-1 decreases cardiomyocyte diastolic stiffness in MCT-induced PAH

We have previously shown that rhNRG-1 improves diastolic function in MCT-induced PAH (15). In order to understand how, we evaluated passive tension in isolated skinned cardiomyocytes from the same animals. Development of PAH was associated with cardiomyocyte increased passive tension at different sarcomeric lengths (Figure 1-A). RhNRG-1 chronic treatment in MCT-induced PAH animals with diastolic dysfunction already present when treatment started (Supplemental Figure 1), decreased RV isolated cardiomyocyte passive tension. LV cardiomyocyte increased passive tension was also observed in MCT-induced PAH and was similarly decreased by rhNRG-1 treatment (Supplemental Figure 2). Interestingly, control animals treated with rhNRG-1 also showed decreased passive tension in the highest measured sarcomeric length in both ventricles. Cardiomyocytes isolated from MCT animals showed increased active tension development compared with control animals, in the RV (Figure 1-B), while rhNRG-1 therapy attenuated this increase.

2.2 Chronic treatment with rhNRG-1 improves calcium signalling and attenuates the expression of cardiac remodelling-associated genes

In order to understand how diastolic function could be modulated by rhNRG-1, we evaluated the effect of rhNRG-1 chronic treatment on two of the main sarcomeric calcium regulation proteins, sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA)2a and phospholamban (PLB). PAH-associated RV diastolic stiffness was paralleled by a decrease in PLB phosphorylation, which was attenuated with rhNRG-1 treatment (Figure 2-A). The expression of Serca2a was similar in the different experimental groups (Figure 2-B). Increased overload of the RV in MCT-induced PAH was associated with altered expression of several remodelling-associated genes. MYH6 gene expression, which encodes the myosin heavy chain α isoform (α-MHC), was decreased in the RV of animals with MCT-induced PAH, while the expression of MYH7 gene, which encodes the myosin heavy chain β isoform (β-MHC) was

increased (Figure 2-C). This resulted in a significant shift of the MYH7/6 ratio. Furthermore, matrix remodelling associated genes, namely collagen alpha-2(I) chain (Col1a2) and collagen alpha-3(I) chain (Col3a1) (Figure 2-D) and skeletal muscle alpha actin (ACTA1) expression were upregulated in the RV from MCT animals (Figure 2-E). Chronic treatment with rhNRG-1 was able to attenuate the MYH7/6 ratio, and the expression of ACTA1, Col3a1 and Col1a2. Changes in remodelling-associated gene expression significantly correlated with hypertrophy and filling pressures of the RV (Supplemental Figure 3).

2.3 Chronic treatment with rhNRG-1 decreases RV remodeling in mild and severe pulmonary arterial banding (PAB)-induced pressure overload without compromising cardiac function

In the present study, we used a mild and severe PAB constriction in order to analyze the rhNRG-1 cardiac-specific effects on diastolic modulation in two different levels of RV pressure overload without PAH. Right heart catheterization showed that only the severe PAB (sPAB) group developed signs of RV failure, with decreased heart rate (HR; Figure 3-A) and ejection fraction (EF; Figure 3-B). RV filling pressures (end-diastolic pressure - EDP; Figure 3-C) and RV dilation (end-diastolic volume - EDV; Figure 3-D) were increased also only in the sPAB group. Additionally, diastolic dysfunction was also present in this group, as measured by tau (τ; Figure 3-E) and end-diastolic elastance (Eed; Figure 3-F, G and H). Although chronic administration of rhNRG-1 did not reduce filling pressures or relaxation time (Figure 3-C and E), it prevented RV dilation and diastolic stiffness (Figure 3-D, F and H) in sPAB animals, potentially contributing to improved diastolic function of the RV. Similar to the RV, LV function was compromised only in the sPAB group with lower end-systolic pressure (ESP) and EDP, suggestive of LV unloading and increased stiffness, and a higher τ, suggestive of compromised relaxation in this group of animals. RhNRG-1 treated animals showed no differences in ESP and EDP compared to sham animals, while τ remained increased (Supplemental Figure 4).

RVH was inversely proportional to the size of the constriction, once the Fulton index was increased in animals subjected to mild PAB (mPAB) (15) and further increased in animals subjected to sPAB. RVH was confirmed by increased cardiomyocyte cross-sectional area (CSA) and interstitial fibrosis, which was also increased in the mPAB group (15) and to a greater extent in the sPAB group (Supplemental Figure 5). RhNRG-1 treatment attenuated RV structural changes, by decreasing the Fulton index in

mPAB animals (15), and cardiomyocyte CSA and fibrosis in both PAB models. Decreased RV remodeling was evident without changing afterload (Figure 3-I), and without compromising overall cardiac function. In fact, RV contractility was preserved (Figure 3-J), as well as EF (Figure 3-B).

2.4 RhNRG-1 attenuates cardiomyocyte diastolic stiffness in mild and severe PAB-induced RV pressure overload

To determine, if the above-mentioned effects of rhNRG-1 on isolated cardiomyocyte passive tension were also observed independently of decreased afterload through pulmonary vascular effects, we performed the same experiments in the RV of PAB animals with mild and severe RV remodelling (16). There was an increase in passive tension in both mild and severe constriction animals (Figure 4-A-C), that was correlated with main structural and functional parameters of diastolic function (Supplemental Figure 6). As in MCT-induced RV hypertrophy, chronic treatment with rhNRG-1 decreased isolated cardiomyocyte passive tension in both mild and severe PAB. Also, cardiomyocytes isolated from mPAB animals showed increased active tension development compared with Sham animals, while sPAB cardiomyocyte active tension was decreased, revealing a depressed contractile function of the cardiomyocytes in this group. Contrary to the MCT protocol, rhNRG-1 did not modulate intrinsic active tension in the PAB models (Figure 4-D).

2.5 Expression of remodeling-associated gene expression in the RV from animals PAB-induced RV hypertrophy animals

Increased overload of the RV in PAB animals was also associated with altered expression of several remodelling-associated genes. ACTA1 expression was upregulated in the RV from mild and severe PAB animals (Figure 5-A and B). MYH6 gene expression was decreased in the RV of animals with severe PAB, while the expression of MYH7 gene was increased (Figure 5-C). Furthermore, COL3A1 was also increased in severe PAB (Figure 5-D). Chronic treatment with rhNRG-1 was able to attenuate the expression of ACTA1 in mild PAB-induced RV hypertrophy.

3. Discussion

In this study we found that rhNRG-1 chronic treatment has beneficial cardiac-specific effects on the passive properties of cardiomyocytes from the RV in PAH. Also, we demonstrated that rhNRG-1 decreased RV intrinsic diastolic stiffness, through the improvement of calcium handling and cardiac remodelling signalling. To our knowledge this is the first report of the effects of rhNRG-1 treatment in RV sarcomeric stiffening in PAH.

In PAH the RV adapts to increased overload, in a compensatory stage, through hypertrophy, with increased RV mass and increased contractility (17). If overload surpasses its capacity to adapt, the RV suffers maladaptive remodelling and begins to fail, leading to decreased cardiac output and ultimately death (18). RV dysfunction is the main determinant of PAH prognosis (19), and recent studies have shown that RV diastolic dysfunction is also present in PAH patients and is associated with the disease severity (2, 3). These patients present increased RV cardiomyocyte hypertrophy and fibrosis, as well as increased cardiomyocyte tension development, reduced calcium sensitivity, and increased RV cardiomyocyte passive stiffness. Thus, therapies focusing on RV diastolic stiffness should effectively improve the prognosis of patients with PAH (3). In line with this, rhNRG-1 was able to attenuate RV sarcomeric passive stiffness, an important factor for RV diastolic impairment in PAH. Indeed, we observed an approximately 2-fold higher RV sarcomeric stiffness over the range of sarcomere lengths in MCT-induced PAH compared with control subjects, and rhNRG-1 therapy attenuated this effect. The advantage of the single RV cardiomyocyte approach is that RV sarcomeric function (the contractile apparatus of the RV cardiomyocytes) can be investigated in detail without the confounding effects of hypertrophy, fibrosis, or calcium handling. Additionally, RV diastolic stiffness in PAH coincided with increased RV contractility and force-generating capacity of cardiomyocytes (active force) as we observed in MCT animals, and rhNRG-1 was able also to decrease this effect.

In PAH, RV diastolic stiffening precedes RV failure (20) and it is associated with sarcomeric protein changes, including titin, troponin I, and myosin binding protein C hypophosphorylation, while no significant changes occur in most of the remaining calcium handling proteins (21). In the experimentally overloaded RV, Serca2a and PLB expression and activity are compromised (22-25). However, in our study just PLB activity seems to be affected. Despite that, care should be taken when

interpreting our results, since sevofluorane (used in our protocol) seems to affect Serca2a and PLB levels in the rat RV (26). In previous studies, NRG-1 increases Serca2a activity through PLB phosphorylation, improving calcium uptake (27). In agreement, we observed a recovery of PLB hypophosphorylation in the RV of MCT animals, when treated with rhNRG-1. This finding suggests that the role of NRG-1 in the diastolic dysfunctional RV might be dependent on PLB phosphorylation. Despite that, we did not find any changes in the phosphorylation or in the expression levels of PLB and Serca2a in the RV of animals submitted to PAB-induced pressure overload (Supplemental Figure 7). The lack of differences suggests that the degree of RV dysfunction observed in both the mild and severe PAB models is not at the same level as the dysfunction observed in the MCT-induced PAH animal model.

We also observed decreased mRNA expression of both type I and type III collagen in the RV in MCT rats treated with rhNRG-1, which might prove to be beneficial, as increased RV collagen mRNA expression is associated with the development of PAH (4, 28, 29). This is consistent with previous studies that have shown that treatment with NRG-1 (30) and gene transfer of human NRG-1 (31) improve LV diastolic function in angiotensin-II induced heart failure (30) and diabetic cardiomyopathy (31), through decreased collagen gene expression and synthesis (32). Furthermore, we have previously shown that rhNRG-1 decreases RV fibrosis in MCT-induced PAH and PAB-induced RV hypertrophy (15), and also in PAB-induced RV dysfunction (severe PAB), as shown in the present manuscript. Decreased hypertrophy observed in MCT animals treated with NRG-1 was associated with a decrease in the gene expression of ACTA1 and an attenuation in the shift of MYH7/6 ratio gene expression, as previously shown in diabetic cardiomyopathy (33). The NRG-1-induced expression of the α MHC isoform (34), encoded by MYH6 gene, might counterbalance the overexpression of the β MHC isoform in the RV present in pulmonary hypertension (35), and contribute to a more efficient actin-myosin interaction at the sarcomeric level. Interestingly, we found that rhNRG-1 treatment in PAB protocol attenuated ACTA1 expression in compensated, but not in decompensated RV hypertrophy, suggesting that the effect of NRG-1 on hypertrophic signalling could differ in distinct stages of cardiac stress. Changes in remodelling-associated gene expression are important triggers for RV diastolic stiffness and, indeed, these alterations are significantly correlated with hypertrophy and filling pressures of the RV in experimental PAH in our study.

Moreover, we demonstrated that chronic treatment with rhNRG-1 decreases RV remodeling in mild and severe PAB-induced pressure overload without compromising cardiac function. As already described (16), the application of a mild constriction resulted in adaptive hypertrophy of the RV (mPAB), with preserved systolic and diastolic function, while application of a severe constriction resulted in maladaptive hypertrophy (sPAB), with chamber dilation and systolic and diastolic dysfunction. Although rhNRG-1 seems to prevent RV dilation in severe PAB, there are no significative functional changes after the treatment. However, we show that rhNRG-1 treatment decreased isolated cardiomyocytes passive tension in both PAB models, being associated with improved RV remodelling, without compromising its function. This is a major benefit of this therapeutic approach, that not only presents specific RV effects, but also comprehends the entire scope of RV adaptation in PAH (17). In fact, it was recently shown that NRG-1 chronic treatment decreases passive tension development in LV myocytes from diabetic mice with diastolic dysfunction (36). These changes were linked to an increased phosphorylation of titin on the more compliant N2Bus-S4010 site and decreased phosphorylation of the stiffer PEVK-S11878 site (36) which overall results in decreased cardiomyocyte passive tension. By itself, the previously published work (36) shows an important molecular mechanism associated to modulation of passive stiffness in diabetes-associated left heart disease, suggesting that a similar action for rhNRG-1 could be present on the RV, explaining also the decreased passive tension development in cells from control animals treated with rhNRG-1. Besides that, rhNRG-1 did not attenuate the expression of remodeling-associated gene expression in the RV from severe PAB animals (Figure 5 and Supplemental Figure 8). Due to their different primary insult, RV adaptation in PAB, compared to MCT model, is significantly different, as previously shown by our group (16), and does not represent the clinical entity of PAH, which could explain this effect. However, the molecular insights related to the cardiac specific effects of NRG-1 on RV dysfunction still need to be investigated in detail and will be the subject of further studies.

Additionally, the observations that rhNRG-1 treatment decreases passive tension in cardiomyocytes isolated from MCT and PAB animals, and that it only affects active tension in animals with MCT-induced PAH, suggest that the effects of rhNRG-1, in this context, are mainly due to the regulation of cardiomyocyte passive properties, and therefore RV diastolic function.

The lack of the direct measurement of calcium transients is a limitation in our study, but taking into account our data, we believe that NRG-1 treatment has a positive role in RV diastolic dysfunction (which was already present when treatment started) specifically improving calcium uptake, through increased PLB phosphorylation, which reduces its inhibitory actions on Serca2a (37). Also, the role of rhNRG-1 in titin modulation on the RV in both animal models would be relevant. However, our study gives the first clue for the possible role of NRG-1 in modulating diastolic function in the overloaded RV, paving the way for future studies focusing on the intracellular pathways associated with our observations.

In the present study, we showed that treatment with rhNRG-1 decreases diastolic stiffness in cardiomyocytes isolated from the RV of animals with MCT-induced PAH, and that this decrease in stiffness was associated with improved remodelling and calcium handling associated signalling. Finally, using animal models of PAB-induced pressure overload, we demonstrated that the effects of rhNRG-1 on isolated cardiomyocytes passive tension are independent from afterload changes. These findings represent a significant step forward towards the understanding of the effects of NRG-1 in the heart, and specifically in the RV and its possible implications in PAH pathophysiology and treatment.

4. Methods

All animal experiments were performed in accordance with 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), and were approved by the ethical committee of the Faculty of Medicine of the University of Porto and the Portuguese Foundation for Science and Technology, and certified by the Portuguese National Authority for Animal Health - DGAV (Direção-Geral de Alimentação e Veterinária) (PTDC/SAU-FCF/100442/2008; 0421/000/000/2013). All animal manipulations were executed by trained researchers, certified with a Laboratory Animal Sciences course according to the Federation of European Laboratory Animal Science Association. Animals were kept in groups of 2-per cage under controlled environment with a 12-h-light-dark cycle at 22°C room temperature, with water and food ad libitum. A detailed method section is available in the online-only Data Supplement.

4.1 Experimental models of MCT-induced PAH and PAB-induced RV pressure overload

Most of the samples used in this study originated from one of our previous works (15). Briefly, seven-week old male Wistar rats (Charles River Laboratories,180-200g) randomly received an injection of MCT (60mg/kg, s.c., Sigma-Aldrich) or an equal amount of vehicle. Two weeks after MCT/vehicle administration, animals were further randomly assigned to receive rhNRG-1 (Peprotech) at a dose of 40 µg/kg (daily, i.p.) or an equal amount of vehicle (0.9%NaCl) for 1 week, resulting in four groups: Ctrl + vehicle (C; n=16); Ctrl + rhNRG-1 (CN; n=14); MCT + vehicle (M; n=24); MCT + rhNRG-1 (MN; n=24). After the treatment period, sample collection was performed for in vitro functional studies and molecular analysis.

Another group of animals was subjected to pulmonary artery banding (PAB), applying a pulmonary artery constriction resulted in two degrees of hypertrophy (16-gauge and 18-gauge needle, for mild and severe PAB, respectively), and submitted to the same randomization, time points, and chronic treatment protocol. So, two weeks after surgery, PAB animals randomly received rhNRG-1 or vehicle for 1 week, resulting in six groups: Sham + vehicle (S; n=8); Sham + rhNRG-1 (SN; n=7); mild PAB + vehicle (mPAB; n=8); mild PAB + rhNRG-1 (mPABN; n=10); severe PAB + vehicle (sPAB; n=12) and severe PAB + rhNRG-1 (sPABN; n=10). After the treatment period, the animals were submitted to hemodynamic evaluation and sample collection was performed for in vitro functional studies and molecular analysis.

4.2 Invasive hemodynamic evaluation

As described (15), animals were intubated and ventilated, and 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, and a bolus injection of hypertonic saline was administered to account for parallel conductance. 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). Following anesthetic overdose, the animals were exsanguinated, and samples collected.

4.3 In vitro studies in isolated skinned cardiomyocytes

RV samples (stored at -80ºC), were defrosted in relax solution, and a biopsy taken, subjected to mechanical disruption, and permeabilized with 0.1% Triton X-100. Under microscopic view (model 1X51, Olympus) and through imaging software (VSL 900B, Aurora Scientific), a single cardiomyocyte was attached to a force transducer (model 403A, Aurora Scientific) and a length controller (model 315C-I, Aurora Scientific). Cell length was digitally adjusted through custom-designed software (series 600A digital controller, Aurora Scientific). Steady-state passive force at increasing sarcomere lengths (1.8 - 2.3 µm) and maximal force development at 2.2 µm were measured (16, 38).

4.4 Quantitative reverse transcription polymerase chain reaction (RT-PCR) and immunoblotting

For RT-PCR, total RNA was extracted using the RNeasy Mini Kit according to manufacturer's instructions, and relative mRNA expression quantified by two-step Real-Time PCR (Step-One™ Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene, and final results presented in Arbitrary Units (AU) and normalized for the control group. All assays were performed in duplicate. Primers used were designed in-house (Supplemental Table 1).

For immunoblotting experiments, RV samples were homogenized in ice-cold RIPA lysis buffer (Cell Signaling Technology) containing protease and phosphatase inhibitors. Extracts were centrifuged at 12000 rpm for 20 min at 4ºC, supernatants collected, total protein concentration determined, and 30µg separated in a 10% SDS-PAGE gel. Proteins were electroblotted onto a 0.2 µm nitrocellulose membrane, blocked with 5% skinned milk and incubated overnight at 4ºC with primary antibodies (Supplemental Table 2). After washing, the membrane was probed with secondary antibodies (IRDye 680LT, Goat-anti-Mouse Ab and IRDye 800CW, Goat-anti-Rabbit Ab, Li-COR Biosciences) and visualized using an Odyssey scanner (infrared imaging system, Li-COR Biosciences).

4.5 Statistical Analysis

The results are presented as the mean±SEM. Statistical analysis was performed using GraphPad Software v7 (GraphPad Software Inc., San Diego, CA, USA). Two-way analysis of variance (ANOVA), with repeated-measures when suitable, was used to analyse most parameters with Tukey's method for post hoc comparisons between groups. A *P*-value of <0.05 was considered to be statistically significant.

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Conflict of Interest

None.

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Figures

Figure 1 – rhNRG-1 treatment decreases passive stiffness and active force development in the cardiomyocytes from MCT-induced PAH animals. (**A**) Passive tension development in isolated skinned cardiomyocytes isolated from the RV of all experimental groups. (**B**) Active tension analysis of cardiomyocytes isolated from the RV of all groups. Symbols and bars represent mean ± SEM, of 7-12 animals per group (29 – 34 cells). *p<0.05, **p<0.01, ***p<0.001 vs C; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs M. Two-way ANOVA repeated measures with Tukey multiple comparison test was used to statistically analyse all the presented parameters. ***Abbreviations: AT** – Active Tension; **C** – control animals treated with vehicle; **CN** – control animals treated with rhNRG-1; **M** – monocrotaline animals treated with vehicle; **MN** – monocrotaline animals treated with rhNRG-1; **rhNRG-1** – recombinant human neuregulin-1; **RV** – right ventricle.*

Figure 2 – rhNRG-1 treatment improves calcium handling signalling and attenuates the expression of remodelling associated genes in the RV from MCT-induced PAH animals. (**A**) Densitometric analysis of PLB phosphorylation at Ser16/Thr17 in RV tissue from all experimental groups. (**B**) Densitometric analysis of Serca2A expression. (**C**) MYH6 and MYH7 mRNA expression (**D**) Collagen 1 and 3 mRNA expression. (**E**) ACTA1 mRNA expression. Symbols and bars represent mean ± SEM, of 6-11 animals per group. *p<0.05 vs C; $^{\#}$p<0.05 vs M. Two-way ANOVA with Tukey multiple comparison test was used for all presented parameters. Acronyms are the same as in Figure 1. ***Abbreviations: ACTA1** – skeletal muscle alpha actin; **C** – control animals treated with vehicle; **CN** – control animals treated with rhNRG-1; **Col1a2** –* collagen alpha-2(I) chain*; **Col3a1** –* collagen alpha-3(I) chain*; **GAPDH** – Glyceraldehyde-3-Phosphate Dehydrogenase; **M** – monocrotaline animals treated with vehicle; **MN** – monocrotaline animals treated with rhNRG-1; **MYH** – myosin heavy chain; **PLB** – phospholamban; **rhNRG-1** – recombinant human neuregulin-1; **RV** – right ventricle; **Serca2A** – sarcoplasmic reticulum Ca2+-ATPase 2a.*

Figure 3 – rhNRG-1 did not improve RV hemodynamic function in mild and severe PAB-induced RV pressure overload. (**A-F**) HR, EF, EDP, EDV, Tau and Eed analysis in all experimental groups. (**G**) Representative RV pressure-volume (PV) loops from mPAB groups (left) and respective diastolic pressure volume relationships (right). (**H**) Representative RV pressure-volume (PV) loops from sPAB groups (left) and respective diastolic pressure volume relationships (right). (**I**,**J**) ESP and Ees analysis in all experimental groups. Symbols and bars represent mean ± SEM, of 7-12 animals per group. *p<0.05, **p<0.01, ***p<0.001 vs S; $^{\#}$p<0.05, $^{\#\#}$p<0.01 vs mPAB. Two-way ANOVA with Tukey multiple comparison test was used to statistically analyse all the presented parameters. ***Abbreviations: EDP –** end-diastolic pressure; **EDPVR –** end-diastolic pressure-volume relationship; **EDV –** end-diastolic volume**; Eed –** end-diastolic elastance; **EF –** ejection fraction; **ESP –** end-systolic pressure; **Ees –** end-systolic elastance; **HR –** heart rate; **mPAB** – mild pulmonary artery banding animals treated with vehicle; **mPABN** – mild pulmonary artery banding animals treated with rhNRG-1; **rhNRG-1** – recombinant human neuregulin-1; **RV** – right ventricle; **S** – sham animals treated with vehicle; **SN** - sham animals treated with rhNRG-1; **sPAB** – severe pulmonary artery banding animals treated with vehicle; **sPABN** – severe pulmonary artery banding animals treated with rhNRG-1; **t –** isovolumic relaxation time constant.*

Figure 4 – rhNRG-1 treatment decreases passive stiffness in RV cardiomyocytes of mild and severe PAB-induced RV pressure overload. (**A-C**) Passive tension development in isolated skinned cardiomyocytes isolated from the RV of all experimental groups. (**D**) Active tension analysis of cardiomyocytes isolated from the RV of all groups. Symbols and bars represent mean ± SEM, of 7-12 animals per group (18-43 cells). *p<0.05, **p<0.01, ***p<0.001 vs S; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs mPAB; §p<0.05, §§§p<0.05 vs sPAB. Two-way ANOVA with Tukey multiple comparison test was used to statistically analyse all the presented parameters. ***Abbreviations: AT** – Active Tension; **mPAB** – mild pulmonary artery banding animals treated with vehicle; **mPABN** – mild pulmonary artery banding animals treated with rhNRG-1; **rhNRG-1** – recombinant human neuregulin-1; **RV** – right ventricle; **S** – sham animals treated with vehicle; **SN** – sham animals treated with rhNRG-1; **sPAB** – severe pulmonary artery banding animals treated with vehicle; **sPABN** – severe pulmonary artery banding animals treated with rhNRG-1.*

Figure 5 – mRNA expression of remodeling associated genes in the RV of PAB-induced hypertrophy animals. (**A,B**) ACTA1 mRNA expression in mild and severe PAB animals. (**C**) MYH6 and MYH7 mRNA expression in severe PAB animals. (**D**) COL3A1 mRNA expression in severe PAB protocol. Symbols and bars represent mean ± SEM, of 7-12 animals per group. *p<0.05, **p<0.01, ***p<0.001 vs S. Two-way ANOVA with Tukey multiple comparison test was used to statistically analyze all the presented parameters. ***Abbreviations: ACTA1** – skeletal muscle alpha actin; **Col3a1** – collagen alpha-3(I) chain; **GAPDH** – Glyceraldehyde-3-Phosphate Dehydrogenase; **mPAB** – mild pulmonary artery banding animals treated with vehicle; **mPABN** – mild pulmonary artery banding animals treated with rhNRG-1; **MYH** – myosin heavy chain; **rhNRG-1** – recombinant human neuregulin-1; **RV** – right ventricle; **S** – sham animals treated with vehicle; **SN** - sham animals treated with rhNRG-1; **sPAB** – severe pulmonary artery banding animals treated with vehicle; **sPABN** – severe pulmonary artery banding animals treated with rhNRG-1.*