TRPM4 modulates right ventricular remodelling under pressure load accompanied with decreased expression level

Short title: Right ventricular hypertrophy is modulated by TRPM4

Authors: Wiebke Frede^{1,2}, Rebekka Medert², Tanja Poth³, Matthias Gorenflo¹, Rudi Vennekens⁴, Marc Freichel², Sebastian Uhl^{1,2}

¹ Department Pediatric and Congenital Cardiology, University Medical Center, Heidelberg, Germany

² Institute of Pharmacology, University Medical Center, Heidelberg, Germany

³ Center for Model System and Comparative Pathology, University Medical Center, Heidelberg, Germany

⁴ Laboratory of Ion Channel Research, Department of Cellular and Molecular Medicine, KU Leuven, Leuven, Vlaams Brabant, Belgium

Corresponding author: Sebastian Uhl, Institute of Pharmacology, University Medical Center, Heidelberg, Germany, phone: 0049/6221-54-86861, fax: 0049/6221-54-8644. email: sebastian.uhl@med.uni-heidelberg.de

Category of the manuscript: Original article

Total word count: 6080

Abstract

Aims: Survival of patients with congenital heart defects including increased right ventricular pressure load (i.e. Tetralogy of Fallot) or pulmonary hypertension is dependent on the function of the right ventricle (RV). RV remodelling has several effects with progressive transition from compensated status to heart failure. Transient receptor potential melastatin 4 (TRPM4) forms cation channels expressed in myocardium, which was shown to modulate cardiac remodelling in the left ventricle of mice. Aim of this study was to identify the role of TRPM4 for contractile function and remodelling of the right ventricle in a rat model of right ventricular pressure load.

Methods and Results: We performed experiments with untreated rats and under Monocrotaline (MCT)-induced pressure load comparing wild type ($Trpm4^{+/+}$) and TRPM4-deficient ($Trpm4^{-/-}$) rats. RV function was characterized by echocardiography and contractility measurements of isolated papillary muscles. RV hypertrophy was investigated by echocardiography and by determination of hypertrophy indices. Pulmonary arterial remodelling was evaluated by echocardiography and histology. TRPM4 protein expression in RV of human, rat and mouse was detected by Western blot and quantified in rat. TRPM4 proteins were detected in RV myocardium of rat and mouse, which were not detectable in TRPM4-deficient animals. Proteins of the same size were found in RV of a pediatric patient with Tetralogy of Fallot. In untreated status, $Trpm4^{+/+}$ and $Trpm4^{-/-}$ rats showed comparable RV contractile function and dimensions. Under pressure load (42 days after Monocrotaline-injection), RV hypertrophy was significantly increased in $Trpm4^{-/-}$ rats in comparison to $Trpm4^{+/+}$ controls, whereas MCT-mediated alterations in cardiac contractility and pulmonary arterial remodelling were not affected by TRPM4 inactivation in rats. Finally, TRPM4 protein expression in RV was drastically reduced in MCT-treated rats whereas left ventricle of the same animals showed no alteration in TRPM4 expression.

Conclusion: Right ventricular pressure load evoked by MCT treatment in rats leads to a prominent downregulation of TRPM4 protein expression in the right ventricle and complete deletion of TRPM4 expression aggravates right ventricular hypertrophy. Thus, therapeutic modulation of TRPM4 expression and activity might represent a novel approach to target right ventricular remodelling in patients with pulmonary hypertension or otherwise loaded RV.

Translational Perspective: Detecting TRPM4 also in pediatric right ventricular myocardium, this channel has potential influence on RV remodelling under pressure loaded right heart in human. Thus, TRPM4 modulation could serve as pharmacological approach in such patients.

Keywords:

TRPM4, cardiac hypertrophy, right ventricular failure, Monocrotaline, pulmonary hypertension

1. Introduction

1.1 Pulmonary hypertension and right ventricular remodelling

Ventricular remodelling appears as a cardiac adaption during chronic cardiovascular stress such as increased afterload or aberrant neurohumoral stimulation. Remodelling processes can be adaptive or maladaptive with morphological, functional and molecular characteristics differing between the left and right ventricle. They alter protein expression pattern, metabolism and capillary density, promote cardiac hypertrophy and fibrosis, as well as elevate apoptosis. Especially in the right heart, these processes are poorly understood yet¹⁻³. An increased pressure load on the right heart can appear due to specific congenital heart defects (e.g. Tetralogy of Fallot or pulmonary valvular/arterial stenosis) and under pulmonary hypertension leading to right ventricular remodelling, which can subsequently end in dysfunction and increased mortality⁴. It is to emphasize that the survival of patients with pulmonary arterial hypertension (PAH) is dependent on the right ventricular function and its (mal-) adaption to the increased afterload¹. New therapeutic strategies that interfere with right heart remodelling could improve this (mal)adaptive response and, consequently, the cardiocirculatory status. To investigate right ventricular remodelling evoked by pressure load in rats, application of the pyrrolizidine alkaloid Monocrotaline (MCT) is an established model to induce pulmonary hypertension⁵. Following a single subcutaneous injection, MCT induces a steadily developing pulmonary arterial remodelling that causes increased pulmonary arterial pressure and right ventricular hypertrophy⁶.

1.2 TRPM4

TRPM4 is a Calcium-activated nonselective cation channel that is predominantly selective for sodium and potassium without conductance for calcium⁷. In the heart, TRPM4 is expressed in the atrium and ventricle with abundance in cardiomyocytes and specialised cells of the cardiac conductive system⁸. TRPM4 is also present in endothelial and smooth muscle cells⁹⁻¹¹. TRPM4-mediated currents are inhibited by intracellular ATP and activated by elevated intracellular calcium concentration^{12,13}. An inhibitory modulation of TRPM4 on L-type calcium current during action potential has been shown¹⁴.

Previous studies described the role of TRPM4 for the development of left ventricular hypertrophy in different models. Following chronic Angiotensin II treatment, mice with cardiac-specific deletion of TRPM4 develop augmented left ventricular mass compared to wild type mice. This finding correlates with increased Calcineurin activity, which mediates cardiac hypertrophy¹⁵. Further, mice lacking TRPM4 exhibit an age-dependent increase in heart weight/body weight ratio, a phenomenon attributed to increased catecholamine levels¹⁶. TRPM4 mRNA is increased in hypertrophied hearts of spontaneously hypertensive

rats, but it remains unclear whether TRPM4 overexpression is causative or adaptive for the left ventricular hypertrophy in this model¹⁷. To date, the influence of TRPM4 regarding right ventricular hypertrophy development has yet to be reported.

Recently, we observed that TRPM4-deficient mice develop increased left ventricular inotropy under β -adrenergic stimulation both *in vivo* and *in vitro*^{14,18}, but the relevance of TRPM4 for right ventricular contractile function has not been investigated.

In this study, we investigated the consequences of chronic right ventricular pressure load on changes in TRPM4 protein expression, contractile function and cardiac hypertrophy development following application of MCT in *Trpm4*^{+/+} (wild type) and TRPM4-deficient (*Trpm4*^{-/-}) rats.

2. Materials and Methods

2.1 Animals

Experiments with rats were performed on male *Trpm4*^{+/+} and *Trpm4*^{-/-} littermate rats at the age of 14-15 weeks. Heterozygote offspring resulted from crossing of female wild type Sprague Dawley and male TRPM4-deficient rat. Homozygote littermates were obtained in second generation by crossing of TRPM4 heterozygote offspring. *Trpm4*^{-/-} rat was generated by Zinc Finger Nucleases interacting with the genetic sequence encoding the pore of TRPM4. This led to a deletion over Exon 18-19 (514 bp including intron 18-19) in one rat, which was then used for further breeding. The genetic deletion of TRPM4 was checked on gene and protein level¹⁹. Euthanasia of rats was performed via Pentobarbital 600 mg/kg i.p. and CO₂-inhalation. Experiments with mice were performed on adult male *Trpm4*^{-/-} mice and C57Bl/6 wild type controls at the age of 9-15 weeks of age. Generation of *Trpm4*^{-/-} mice is described in detail elsewhere²⁰. Euthanasia of mice was performed via neck dissection. All animal experiments were performed in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and German legislation on protection of animals and were approved by the Ethics Review Board of University of Heidelberg (reference number 35-9185.81/G-180/16).

2.2 Human tissue

Human specimen were obtained intraoperatively based on medical indication in consent with vote S-390/2011 of the ethical committee of University of Heidelberg, Germany, in consent to the principles outlined in the Declaration of Helsinki and with informed consent of the patient and the legal guardian, respectively.

2.3 Monocrotaline treatment

Monocrotaline (Crotaline, MCT, Sigma-Aldrich, Taufkirchen, Germany) was dissolved in HCL 1N and buffered with NaOH to a pH of 7,40. The injection of Monocrotaline (60mg/kg body weight) was performed subcutaneously in a single application. Control animals of the same age were not injected. The rats were divided in four groups: Trpm4^{+/+} littermates without treatment (untreated healthy rats), Trpm4^{+/+} littermates with treatment of MCT, Trpm4^{-/-} rats without treatment (untreated healthy rats), Trpm4^{-/-} rats with treatment of MCT.

2.4 Microsomal Preparation and Western Blot

Microsomal membrane preparation:

Ventricular tissue was homogenized with a 2 ml tissue grinder (Tenbroeck Tissue Grinders, Wheaton) in 1 ml lysis buffer (100 mM TRIS-HCl; 1 mM MgCl₂; pH 8,0) supplemented with protease inhibitor cocktail (1 mM Iodacetamide; 1 mM Phenantholin; 0,1 mM PMSF; 1 µg/ml Antipain; 1 µg/ml Leupeptin; 0,7 µg/ml Pepstatin; 1 mM Benzamidine; 0,3 µM Aprotinin). Lysate was filled up to a volume of 3 ml with lysis buffer and frozen at -80°C for 20 min. Afterwards the ventricular tissue lysate was thawed on ice for 1h. Samples were diluted in 15 ml of sucrose buffer (0,25 M sucrose; 10 mM TRIS-HCl; pH 7,4) and centrifuged for 30 min at 6000g. Supernatant was transferred to 26 ml ultracentrifuge bottle (Beckman, 355618) and centrifuged at 50.000g for 1 h 45 min. Supernatant was removed and microsomal membrane pellet was diluted in 200 µl sucrose buffer. Protein concentration was determined by BCA assay (ThermoFisher Scientific USA; 23227). Microsomal membrane fractions were supplemented with Laemmli buffer (60 mM TRIS-HCl; 10 % (v/v) Glycerol; 5 % (v/v) β-mercaptoethanol; 4 % (w/v) SDS; 0,005 % (w/v) Bromophenol blue; pH 6,8) and incubated at 60°C for 20 min.

Immunoblotting:

Microsomal membrane fractions (80 µg/well) were loaded on a 10 % or 4-12 % Bis-Tris Plus gel (Invitrogen USA; NW04122) and resolved at 130 V for 90 min followed by electroblotting onto a 0,45 µm Protran nitrocellulose membrane (GE Healthcare; A10195922) at 12 mV for 60 min. The blot was blocked in 5 % non-fat milk with TBST (50 mM TRIS-HCl; 150 mM NaCl; 0,1 % (v/v) Tween-20; pH 7,5). Blots were incubated in anti-GAPDH 1:500 (Acris USA; ACR001P), anti-Calnexin 1:1000 (ab1003 provided by Veit Flockerzi) or anti-TRPM4 1:100 (ab578 provided by Veit Flockerzi²⁰) at 4 °C overnight. Blots were washed with TBST and incubated in horseradish peroxidase-conjugated anti-rabbit 1:50000 (GE Healthcare; NA9340V) or anti-mouse 1:50000 (Sigma-Aldrich, A5278) at RT for 2h. Blots were washed and incubated in SignalFire Elite ECL (Cell Signaling USA; 12757S) for 1 min.

Chemoluminescence was detected by digital imaging (GE Healthcare, ImageQuant LAS 4000 mini) and densitometry was measured by ImageJ software.

2.5 Histology

Lungs were separated, rinsed with sodium chloride (NaCl 0,9%) and fixed in paraformaldehyde. For evaluation of pulmonary arterial media thickness, 5 µm sections of lungs were prepared and stained with Elastica-van-Gieson. Sections were scanned with 400x magnification and analysed electronically with Image scope software. The thickness of the pulmonary arterial media was measured in two groups of peripheral pulmonary arteries (25-50 µm and 51-100 µm external diameter, respectively) and measured as distance from Lamina elastica interna to Lamina elastica externa. External diameter was measured as a mean of two independent measurements. Media thickness was analysed for each artery as mean of 4 measurements, and 50-60 vessels were analysed for each animal. Relative pulmonary arterial media thickness (%) was calculated as arterial media thickness/external diameter x 100.

2.6 Contractility measurements in intact papillary muscles isolated from right ventricle

Contractility measurements were performed on isolated papillary muscles from right ventricle. Rats were sacrificed (see section 2.1) and the heart was quickly removed and transferred to a petri dish containing Krebs-Henseleit (KH) solution with 25 mM 2,3-butanedione monoxime (BDM) at 10 °C gassed with 100 % O₂. Under a stereoscopic microscope the anterior and posterior papillary muscles of the right ventricle were dissected, whereas parts of ventricular wall and tricuspid valve were preserved to attach silk sutures. Papillary muscles were fixed vertically in an organ bath chamber (IOA 5300, Föhr Medical Instruments GmbH, Germany) filled with KH solution containing (in mM): 118 NaCl; 4.7 KCl; 24.88 NaHCO₃; 1.2 CaCl₂; 1.64 MgSO₄; 1.18 KH₂PO₄; 5,56 Glucose; 1 sodium pyruvate. The chambers were gassed with carbogen all the time. Temperature of solution was maintained at 32°C. After transfer in the organ bath chamber, electrical pacing was started immediately with impulses of 2ms duration at a frequency of 0,5 Hz. During an equilibration period of 45 minutes the muscle preparation was stretched to a length where twitch force reached its maximal value. Twitch force was recorded by a force transducer (TIM1030, Föhr Medical Instruments GmbH, Germany). To investigate twitch characteristics, absolute force as well as time-dependent parameters such as time to peak (TTP) and time of half relaxation (R₅₀) were analysed (Supplementary figure 2A). For recording and analysis the Chart® software (ADInstruments) was used.

To investigate force-frequency relation (FFR), contractility was investigated in the frequency range between 0.1 and 3 Hz. Normalised twitch force refers to basal twitch force at 0.5 Hz. β -adrenergic inotropic effects were characterised by application of Isoprenaline into the organ bath chamber in additive manner. Its concentration was increased after a contractile response had been attained for the previous.

2.7 Echocardiography

Transthoracic echocardiography was performed on day 42 after MCT injection and untreated control rats were matched in gender, age and strain. Sedation of rats was performed using continuous Isoflurane (1,5-3,5 vol%, depending on depressant effect) inhalation via vaporizer under spontaneous breathing. Rats were placed on a heating plate (38°C), fixed and shaved. Echocardiography was performed with Visualsonic Vevo 2100® and a 13–24 MHz transducer under ECG-, temperature- and respiration- control.

Transthoracic 2-dimensional, M-mode and Doppler imaging were performed according to standards of European Association of Cardiovascular Imaging²¹. For evaluation of RV morphology the following parameters were assessed during systole (s) and diastole (d): RV free-wall thickness on midpapillary level in M-mode (RVAW), RV free-wall thickness in the outflow tract in M-mode (RVAWOT), diameter of RV diastolic and systolic on midpapillary level in M-mode (RVD), diameter of RV outflow tract in M-mode (RVOTD). For evaluation of RV function, the following parameters were assessed: shortening fraction (SF) as percent change in relation systolic to diastolic RV cavity dimensions with calculation $SF (\%) = (RVDd - RVDs) / RVDd \times 100$. Tricuspid annular plane systolic excursion (TAPSE) with M-mode in four chamber view, RV fractional area change (RVFAC) as relation of RV area in systole to diastole in four chamber view with calculation $RV FAC (\%) = (RV_{diastolic\ area} - RV_{systolic\ area}) / RV_{diastolic\ area} \times 100$. The velocity of the maximal tricuspid annular systolic motion (RV S') was analysed using the tissue Doppler mode in four chamber view. RV S' was also shown to estimate pulmonary arterial blood pressure²². For further evaluation of pulmonary arterial blood pressure pulmonary arterial acceleration time (PAAT) was assessed from the onset of systolic flow to peak pulmonary arterial velocity in pulsed wave Doppler mode in the proximal main pulmonary artery. PAAT was normalised to cardiac cycle length (CL) as measured within the same cycle.

For evaluation of LV morphology and function the following parameters were assessed in M-mode on mid-papillary level in systole (s) and diastole (d): interventricular septum thickness (IVS), LV anterior wall thickness in systole and diastole (LVAW), LV posterior wall thickness in systole and diastole (LVPW), diameter of LV end-diastolic and end-systolic on midpapillary level in M-mode (LVESD and LVEDD), fractional shortening (FS) as percental change in

relation systolic to diastolic LV cavity dimensions with calculation $FS (\%) = (LVEDD - LVESD) / LVEDD \times 100$, ejection fraction (EF) as percental change in relation systolic to diastolic LV cavity dimensions with calculation $EF (\%) = (LV \text{ Volume}_{\text{diastolic}} - LV \text{ Volume}_{\text{systolic}}) / \text{Volume}_{\text{diastolic}} \times 100$, stroke volume (SV) as difference of enddiastolic volume - endsystolic volume. Endsystolic volume was calculated by the echocardiographic software with end major and end area in systole and diastole, respectively using the formula $\text{Volume}_{\text{endsystolic}} = (4\pi/3) \times (\text{major}_{\text{endsystolic}}/2) \times (\text{area}_{\text{endsystolic}} / (\pi(\text{major}_{\text{endsystolic}}/2)))^2$. Enddiastolic volume was calculated accordingly.

2.8 Statistical analysis

Absolute values are given as mean $\pm$ SEM. Normalised values of the contractile measurements of papillary muscles refer to basal twitch force. Statistical analysis of two groups was performed by t-test. Variance analysis of more than two groups was performed by ANOVA test using Origin Software. A p-value less than 0.05 was considered significant.

3. Results

3.1 TRPM4 protein expression in human, mouse and rat

To identify TRPM4 protein in right ventricular tissue, we performed western blot analysis using microsomal protein fractions and the affinity-purified rabbit polyclonal antibody Ab578 directed against the mouse TRPM4 amino-terminus²⁰. A 136 kDa protein was detected in the right ventricle of wild type mice and rats, whereas these proteins were not detected in samples from *Trpm4*^{-/-} mice or rats, respectively. A protein of comparable size was found in a right ventricular sample of a child with Tetralogy of Fallot (Figure 1).

3.2 Development of right ventricular hypertrophy under MCT-induced pressure load is enhanced in TRPM4-deficient rats

42 days after MCT treatment, both *Trpm4*^{+/+} and *Trpm4*^{-/-} rats showed increased right ventricular mass compared to untreated control groups (Figure 2). MCT-treated rats showed increased absolute weight of isolated right ventricular wall as well as in relation to tibia length and left ventricular weight (Figure 2 A-C). This effect on right ventricular hypertrophy development was significantly augmented in hearts of *Trpm4*^{-/-} rats compared to *Trpm4*^{+/+} controls. In contrast, left ventricular mass was not altered by MCT treatment (Supplementary Figure 1A and B).

Analogue to allometric analysis, we detected changes in right ventricular parameters using echocardiographic measurements (Figure 2D). The right ventricular free wall was thickened in MCT-treated animals at the mid-papillary level and right ventricular outflow tract, whereas *Trpm4*^{-/-} hearts showed increased hypertrophy parameters compared to *Trpm4*^{+/+} control

rats. As expected from hypertrophy index analysis, all parameters describing left ventricular dimensions remained unaltered in *Trpm4*^{-/-} hearts compared to MCT-treated wild type controls (Supplementary Figure 1C).

3.3 TRPM4-deficient rats show equivalent pulmonary remodelling after MCT treatment

To exclude altered pulmonary remodelling as a cause for augmented right ventricular hypertrophy development in *Trpm4*^{-/-} rats, we investigated 1) lung weight and 2) media thickness as parameters for MCT-mediated pulmonary remodelling, as well as 3) echocardiographic parameters to estimate pulmonary arterial pressure. MCT treatment augmented lung weight in both genotypes, but there was no difference between *Trpm4*^{-/-} and *Trpm4*^{+/+} rats (Figure 3A). Pulmonary artery media thickness was also increased by MCT treatment without alteration between *Trpm4*^{-/-} rats and *Trpm4*^{+/+} controls (Figure 3B). Figure 3C shows echocardiographic measurement of pulmonary arterial acceleration time (PAAT) and velocity of the maximal tricuspid annular systolic motion (RV S') describing alterations consistent with a rise in pulmonary arterial pressure equivalent in *Trpm4*^{-/-} rat and *Trpm4*^{+/+} controls. In summary, these experimental approaches showed that MCT-induced changes in lung weight and morphology/hemodynamics in the pulmonary arterial system were not different between *Trpm4*^{-/-} and *Trpm4*^{+/+} indicating that animals of both genotypes were challenged to a comparable extent.

3.4 Right ventricular contractile function in untreated and MCT-treated animals does not differ between wild type and TRPM4-deficient rats

To assess cardiac contractile function, we performed contractility measurements on isolated intact right ventricular papillary muscles as well as *in vivo* by echocardiography. *In vitro* measurements with isolated right ventricular papillary muscles from untreated animals showed comparable twitch force parameters in *Trpm4*^{+/+} and *Trpm4*^{-/-} rats under basal stimulation (Figure 4A and B). With increasing pacing frequency (from 0.5 to 2 Hz), we observed a gain of twitch force that corresponds to a positive force-frequency relation in preparations without MCT treatment (Figure 4C; Supplementary Figure 2C). Isoprenaline application induced equivalent positive inotropic effects in preparations from *Trpm4*^{+/+} and *Trpm4*^{-/-} rats (Figure 4D).

After MCT injection, we detected increased contractile properties under baseline conditions, but there were no differences between *Trpm4*^{+/+} and *Trpm4*^{-/-} preparations (Figure 4A and B; Supplementary Figure 2B). *Trpm4*^{+/+} and *Trpm4*^{-/-} papillary muscle preparations developed higher absolute contractile forces after MCT treatment at pacing frequencies between 0.1

and 1 Hz compared to untreated control groups. Under augmentation of the pacing frequency a decrease in twitch force was observed corresponding to a negative force-frequency relation (Figure 4C; Supplementary Figure 2C). Isoprenaline application caused comparable positive inotropic effects in all four groups, with slightly higher contractile level in MCT-treated animals under low Isoprenaline concentrations (Figure 4D; Supplementary Figure 2D).

Echocardiographic measurements in untreated animals of both genotypes showed a comparable right ventricular pump function. After MCT treatment, contractile parameters (TAPSE and RVFAC) were diminished, but without differences between *Trpm4*^{+/+} and *Trpm4*^{-/-} rats (Figure 4E). Left ventricular pump function was unaltered between genotypes and MCT treatment status (Supplementary Figure 1C).

3.5. TRPM4 protein expression is decreased in pressure loaded right ventricle of rats

We performed a quantitative analysis of TRPM4 protein expression by comparing heart samples from untreated animals and rats following MCT treatment. In the right ventricle of MCT-treated animals, TRPM4 protein was significantly reduced (Figure 5A). In contrast to this finding in the right ventricle, TRPM4 protein expression in the left ventricular myocardium from the same animals showed no alterations in expression level of TRPM4 proteins between untreated and MCT-treated rats. This finding argues against an unfavourably direct effect of MCT on TRPM4 expression in cardiac cells.

4. Discussion

Right ventricular remodelling and subsequent right ventricular dysfunction affects morbidity and mortality of patients with loaded right ventricle. TRPM4, a nonselective cation channel in cardiomyocytes, was recently shown as a critical modulator of left ventricular hypertrophy and contractile function^{14,15,23}, but its role for right ventricular remodelling and/or function remains unclear. In this study, we performed experiments on a TRPM4-deficient rat model compared with wild type littermates under physiological conditions as well as under MCT-induced right ventricular pressure load.

We detected TRPM4 proteins in the right ventricular myocardium from mice using a polyclonal antibody directed against the TRPM4 amino-terminus that specifically identifies TRPM4 proteins in tissues from wild type but not TRPM4-deficient mice²⁰. Here, we revealed that this anti-TRPM4 antibody specifically identifies a 136 kDa protein in the rat right ventricle. The specificity of this antibody was confirmed by using protein fractions from

Trpm4^{-/-} rats, in which no detection appeared. Using this antibody in a human right ventricular specimen of a 5 months old child with Tetralogy of Fallot, we detected a protein of the same size as in rat and mouse, a result that proves the expression of TRPM4 in the human right heart.

In our rat model, a single 60 mg/kg MCT dose applied subcutaneously triggered pulmonary remodelling with increased lung weight, elevated pulmonary arterial media thickness and echocardiographic signs for pulmonary arterial hypertension. These MCT-induced effects are congruent with published data²⁴⁻²⁷, and the extent of these pulmonary alterations were comparable in Trpm4^{+/+} and Trpm4^{-/-} rats.

MCT-induced right ventricular hypertrophy turned out to be enhanced in Trpm4^{-/-} animals compared to wild type littermates. Untreated Trpm4^{+/+} and Trpm4^{-/-} rats showed a comparable right ventricular mass, a finding that indicates a role of TRPM4 for cardiac remodelling in the pressure loaded right ventricle but not under unstressed conditions. MCT had no effect on left ventricular mass in Trpm4^{+/+} or Trpm4^{-/-} rats, data that illustrate MCT-evoked pulmonary arterial remodelling induces cardiac hypertrophy selectively in the right ventricle.

The contractility of isolated papillary muscle preparations was increased in MCT-treated rats under baseline conditions compared to untreated control preparations. This result is consistent with a hypertrophic effect in the right ventricle^{3,28}. Under increasing pacing frequencies, muscle preparations of untreated Trpm4^{+/+} and Trpm4^{-/-} rats showed positive inotropic effects (positive force- frequency relation), whereas twitch force declined in MCT-treated preparations by increase in stimulation frequency (negative force- frequency relation). A previous study reported similar results in muscle strip preparations of Wistar rats after MCT treatment²⁸. Isoprenaline application induced positive inotropic effects in right ventricular papillary muscles from all groups, with no differences between Trpm4^{-/-} and littermate controls. These *in vitro* findings describe an MCT-mediated hypertrophic effect with compensated contractile function under resting conditions and a beginning loss of contractile reserve under increased energy consumption in isolated right ventricular myocardium, as observed under increased pacing frequency or pharmacological stimulation. Investigation of contractile function *in vivo* using echocardiography showed that MCT-induced pressure load decreased right ventricular function compared to untreated rats. Left ventricular systolic function was not altered between Trpm4^{+/+} and Trpm4^{-/-} rats or between MCT-treated versus untreated groups. Thus, the impairment of *in vivo* systolic pump function by MCT treatment was limited to the right ventricle.

In summary, we found no difference in contractile function of the right ventricle between *Trpm4*^{-/-} and littermate controls. This observation, especially concerning the equivalent Isoprenaline-mediated twitch force in *Trpm4*^{-/-} and wild type papillary muscle preparations in untreated rats, appears contradictory to published data of left ventricular muscle preparations in mice, where Isoprenaline induced a higher twitch force in left papillary muscles of *Trpm4*^{-/-} mice in comparison to wild type controls¹⁴. We must emphasise that a cardiac phenotype observed in the left heart cannot be extrapolated to the right ventricle without restrictions²⁹. Based on the results of this study in a rat model with MCT-evoked pulmonary arterial remodelling resulting in right ventricular hypertrophy with compensated cardiac contractile status, we cannot exclude that TRPM4 has a potential effect on cardiac contractility in rats under right ventricular decompensation.

Interestingly, TRPM4 protein expression was significantly depressed in the right ventricular myocardium of *Trpm4*^{+/-} rats with pressure load of RV. We excluded a direct toxic effect of MCT on TRPM4 expression, since western blot of the left ventricle from the same animals showed that MCT did not alter TRPM4 expression. These results indicate that TRPM4 expression is dependent on pressure load of the right ventricle. Hence, we speculate that TRPM4 downregulation represents a (mal-) adaptive effect on the increased right ventricle afterload. The difference in right ventricular hypertrophy between MCT-treated *Trpm4*^{+/-} and *Trpm4*^{-/-} rats, despite drastically decreased TRPM4 protein level in the *Trpm4*^{+/-} rats, illustrates the importance of TRPM4 for right ventricular remodelling effects.

Kecskés *et al.* investigated left ventricular hypertrophy in mice under chronic Angiotensin II application to evaluate the contribution of TRPM4 for cardiac hypertrophy. They demonstrated increased cardiac hypertrophy in cardiomyocyte-specific TRPM4-deficient compared to wild type mice. This phenotype was apparently caused by the lack of TRPM4-mediated regulation of the Calcineurin-NFAT-pathway, which consequently increased hypertrophy in *Trpm4*^{-/-} mice. However, there were no changes in detected left ventricle TRPM4 expression following chronic Angiotensin II application¹⁵. In vascular endothelium of spontaneous hypertensive rats, Ding *et al.* observed increased TRPM4 expression under a high salt diet mediating oxidative stress in endothelial cells. These findings suggest a maladaptive effect of TRPM4 for endothelial function³⁰. Same rat model was shown to have increased mRNA level of TRPM4 in myocardium of the left ventricle, where TRPM4 could participate in processes of cardiac arrhythmias¹⁷.

Based on the results of this study, we hypothesise that TRPM4 limits hypertrophic effects in the right ventricle of rats under ventricular pressure load. In the pressure loaded right ventricle, TRPM4 expression decreases compared to physiological level. Thus, the limiting effect of TRPM4 on remodelling pathways vanishes and triggers right ventricle hypertrophy. Further studies need to clarify whether this adaption is maladaptive or beneficial for long-term function of the right heart.

5. Funding

This work was supported by the German foundation of cardiac science (Deutsche Stiftung für Herzforschung (SU, MF, MG), the Transregional Collaborative Research Centre 152 (MF), the DZHK (German Centre for Cardiovascular Research) and the BMBF (German Ministry of Education and Research) (MF).

6. Acknowledgements

We thank C. Matka, M. Ritzal, and H.-P. Gensheimer and the Center for Model System and Comparative Pathology Heidelberg (CMCP, Germany) for expert technical assistance.

7. Conflict of Interest

none declared.

8. References

1. Vonk-Noordegraaf A, Haddad F, Chin KM, Forfia PR, Kawut SM, Lumens J, Naeije R, Newman J, Oudiz RJ, Provencher S, Torbicki A, Voelkel NF, Hassoun PM. Right heart adaptation to pulmonary arterial hypertension: physiology and pathobiology. *J Am Coll Cardiol*. 2013;**62**:D22-33.
2. Friedberg MK, Redington AN. Right versus left ventricular failure: differences, similarities, and interactions. *Circulation* 2014;**129**:1033-1044.
3. van der Bruggen CEE, Tedford RJ, Handoko ML, van der Velden J, de Man FS. RV pressure overload: from hypertrophy to failure. *Cardiovasc Res*. 2017;**113**:1423-1432.
4. Guimaron S, Guihaire J, Amsallem M, Haddad F, Fadel E, Mercier O. Current Knowledge and Recent Advances of Right Ventricular Molecular Biology and Metabolism from Congenital Heart Disease to Chronic Pulmonary Hypertension. *Biomed Res Int*. 2018;**2018**:1981568.
5. Santos-Ribeiro D, Mendes-Ferreira P, Maia-Rocha C, Adao R, Leite-Moreira AF, Bras-Silva C. Pulmonary arterial hypertension: Basic knowledge for clinicians. *Arch Cardiovasc Dis*. 2016;**109**:550-561.
6. Nogueira-Ferreira R, Vitorino R, Ferreira R, Henriques-Coelho T. Exploring the monocrotaline animal model for the study of pulmonary arterial hypertension: A network approach. *Pulm Pharmacol Ther*. 2015;**35**:8-16.
7. Clapham DE. TRP channels as cellular sensors. *Nature* 2003;**426**:517-524.
8. Freichel M, Berlin M, Schurger A, Mathar I, Bacmeister L, Medert R, Frede W, Marx A, Segin S, Londono, JEC. Frontiers in Neuroscience. TRP Channels in the Heart. In: nd, Emir TLR, eds. Neurobiology of TRP Channels. Boca Raton (FL): CRC Press/Taylor & Francis (c) 2018 by Taylor & Francis Group, LLC., 2017:149-185.

- 474 9. Guinamard R, Chatelier A, Demion M, Potreau D, Patri S, Rahmati M, Bois P.
475 Functional characterization of a Ca(2+)-activated non-selective cation channel in
476 human atrial cardiomyocytes. *J Physiol.* 2004;**558**(Pt 1):75-83.
- 477 10. Guinamard R, Rahmati M, Lenfant J, Bois P. Characterization of a Ca2+-activated
478 nonselective cation channel during dedifferentiation of cultured rat ventricular
479 cardiomyocytes. *J Membr Biol.* 2002;**188**:127-135.
- 480 11. Murakami M, Xu F, Miyoshi I, Sato E, Ono K, Iijima T. Identification and
481 characterization of the murine TRPM4 channel. *Biochem Biophys Res Commun*
482 2003;**307**(3):522-8.
- 483 12. Nilius B, Prenen J, Voets T, Droogmans G. Intracellular nucleotides and polyamines
484 inhibit the Ca2+-activated cation channel TRPM4b. *Pflugers Arch.* 2004
485 Apr;**448**(1):70-5.
- 486 13. Ullrich ND, Voets T, Prenen J, Vennekens R, Talavera K, Droogmans G, Nilius B.
487 Comparison of functional properties of the Ca2+-activated cation channels TRPM4
488 and TRPM5 from mice. *Cell calcium* 2005;**37**:267-278.
- 489 14. Mathar I, Kecskes M, Van der Mieren G, Jacobs G, Camacho Londono JE, Uhl S,
490 Flockerzi V, Voets T, Freichel M, Nilius B, Herijgers P, Vennekens R. Increased beta-
491 adrenergic inotropy in ventricular myocardium from Trpm4^{-/-} mice. *Circ Res.*
492 2014;**114**:283-294.
- 493 15. Kecskes M, Jacobs G, Kerselaers S, Syam N, Menigoz A, Vangheluwe P, Freichel M,
494 Flockerzi V, Voets T, Vennekens R. The Ca(2+)-activated cation channel TRPM4 is a
495 negative regulator of angiotensin II-induced cardiac hypertrophy. *Basic Res Cardiol.*
496 2015;**110**(4):43.
- 497 16. Mathar I, Vennekens R, Meissner M, Kees F, Van der Mieren G, Camacho Londono
498 JE, Uhl S, Voets T, Hummel B, van den Bergh A, Herijgers P, Nilius B, Flockerzi V,
499 Schweda F, Freichel M. Increased catecholamine secretion contributes to
500 hypertension in TRPM4-deficient mice. *J Clin Inv.* 2010;**120**:3267-3279.
- 501 17. Guinamard R, Demion M, Magaud C, Potreau D, Bois P. Functional expression of the
502 TRPM4 cationic current in ventricular cardiomyocytes from spontaneously
503 hypertensive rats. *Hypertension (Dallas, Tex : 1979)* 2006;**48**:587-594.
- 504 18. Uhl S, Mathar I, Vennekens R, Freichel M. Adenylyl cyclase-mediated effects
505 contribute to increased Isoprenaline-induced cardiac contractility in TRPM4-deficient
506 mice. *J Mol Cell Cardiol.* 2014 Sep;**74**:307-17.
- 507 19. Bovet-Carmona M, Menigoz A, Pinto S, Tambuyzer T, Krautwald K, Voets T, Aerts
508 JM, Angenstein F, Vennekens R, Balschun D. Disentangling the role of TRPM4 in
509 hippocampus-dependent plasticity and learning: an electrophysiological, behavioral
510 and fMRI approach. *Brain Struct Funct.* 2018 Nov;**223**(8):3557-3576.
- 511 20. Vennekens R, Olausson J, Meissner M, Bloch W, Mathar I, Philipp SE, Schmitz F,
512 Weissgerber P, Nilius B, Flockerzi V, Freichel M. Increased IgE-dependent mast cell
513 activation and anaphylactic responses in mice lacking the calcium-activated
514 nonselective cation channel TRPM4. *Nat Immunol.* 2007 Mar;**8**(3):312-20.
- 515 21. Galderisi M, Cosyns B, Edvardsen T, Cardim N, Delgado V, Di Salvo G, Donal E,
516 Sade LE, Ernande L, Garbi M, Grapsa J, Hagendorff A, Kamp O, Magne J, Santoro
517 C, Stefanidis A, Lancellotti P, Popescu B, Habib G, Eacvi Scientific Documents
518 CommitteeStandardization of adult transthoracic echocardiography reporting in
519 agreement with recent chamber quantification, diastolic function, and heart valve
520 disease recommendations: an expert consensus document of the European
521 Association of Cardiovascular Imaging. *Eur Heart J Cardiovasc Imaging*
522 2017;**18**:1301-1310.
- 523 22. Parasuraman S, Walker S, Loudon BL, Gollop ND, Wilson AM, Lowery C, Frenneaux
524 MP. Assessment of pulmonary artery pressure by echocardiography-A
525 comprehensive review. *Int J Cardiol Heart Vasc.* 2016;**12**:45-51.
- 526 23. Jacobs G, Oosterlinck W, Dresselaers T, Geenens R, Kerselaers S, Himmelreich U,
527 Herijgers P, Vennekens R. Enhanced beta-adrenergic cardiac reserve in Trpm4^(-/-)
528 mice with ischaemic heart failure. *Cardiovasc Res.* 2015;**105**:330-339.

24. Sadamura-Takenaka Y, Ito T, Noma S, Oyama Y, Yamada S, Kawahara K, *et al.* HMGB1 promotes the development of pulmonary arterial hypertension in rats. *PloS one* 2014;**9**:e102482
25. Steven S, Oelze M, Brandt M, Ullmann E, Kroller-Schon S, Heeren T, Tran, LP, Daub S, Dib M, Stalleicken D, Wenzel P, Munzel T, Daiber, A. Pentaerythritol Tetranitrate In Vivo Treatment Improves Oxidative Stress and Vascular Dysfunction by Suppression of Endothelin-1 Signaling in Monocrotaline-Induced Pulmonary Hypertension. *Oxid Med Cell Longev.* 2017;**2017**:4353462.
26. Wilson DN, Schacht SE, Al-Nakkash L, Babu JR, Broderick TL. Resveratrol prevents pulmonary trunk remodeling but not right ventricular hypertrophy in monocrotaline-induced pulmonary hypertension. *Pathophysiology.* 2016 (**4**):243-250.
27. Hardziyenka M, Campian ME, de Bruin-Bon HA, Michel MC, Tan HL. Sequence of echocardiographic changes during development of right ventricular failure in rat. *J Am Soc Echocardiogr.* 2006 (**10**):1272-9.
28. Kogler H, Hartmann O, Leineweber K, Nguyen van P, Schott P, Brodde OE, Hasenfuss G. Mechanical load-dependent regulation of gene expression in monocrotaline-induced right ventricular hypertrophy in the rat. *Circ Res.* 2003;**93**:230-237.
29. Handoko ML, de Man FS, Allaart CP, Paulus WJ, Westerhof N, Vonk-Noordegraaf A. Perspectives on novel therapeutic strategies for right heart failure in pulmonary arterial hypertension: lessons from the left heart. *Eur Respir Rev.* 2010 (**115**):72-82.
30. Ding XQ, Ban T, Liu ZY, Lou J, Tang LL, Wang JX, Chu WF, Zhao D, Song BL, Zhang ZR. Transient Receptor Potential Melastatin 4 (TRPM4) Contributes to High Salt Diet-Mediated Early-Stage Endothelial Injury. *Cell Physiol Biochem.* 2017;**41**(2):835-848.

9. Figure legends

Figure 1: TRPM4 protein is expressed in right ventricular myocardium of human, rat, and mouse but not in TRPM4-deficient animals. Western blot of microsomal membrane protein fractions from right ventricles using antibody specifically against TRPM4 detecting proteins in human, wild type rat and wild type mouse but not in TRPM4-deficient rat and TRPM4-deficient mouse. GAPDH Glycerinaldehyd-3-phosphat-Dehydrogenase, Trpm4^{-/-} TRPM4-deficient, Trpm4^{+/+} wild type.

Figure 2: Inactivation of TRPM4 leads to increased right ventricular hypertrophy in rat under pressure load. Right ventricular weight in absolute (A), in relation to tibia length (B) and weight of left ventricle plus interventricular septum (C) n=10-12/group. Differences between groups were evaluated by ANOVA test (comparing Trpm4^{-/-} vs. Trpm4^{+/+}: *p<0.05, **p<0.01; comparing to untreated: #p<0.05, ##p<0.01). (D) Table of echocardiographic parameters evaluating right ventricular dimensions of untreated animals (left panel) and 42 days after Monocrotaline-injection (right panel). Representative example of RVAW in an untreated (MCT -) and treated (MCT +) Trpm4^{+/+} animal is shown on the left. n= 10-15/group. Differences between groups were evaluated by ANOVA test (comparing Trpm4^{-/-} vs. Trpm4^{+/+}: *p<0.05, **p<0.01; comparing to untreated: #p<0.05, ##p<0.01). AW anterior

574 wall, (e)d (end)diastolic, (e)s (end)systolic, MCT Monocrotaline, *n* number, IVS
 575 interventricular septum, LV left ventricle, OT outflow tract, PW posterior wall, RV right
 576 ventricle, *Trpm4*^{-/-} TRPM4-deficient, *Trpm4*^{+/+} wild type.

577
 578 **Figure 3: Pulmonary remodelling in TRPM4-deficient rats after Monocrotaline-injection**
 579 **is equivalent to wild type animals.** (A) Lung weight in relation to tibia length. *n*= 7-9/group.
 580 Differences between groups were evaluated by ANOVA test ([#]*p*<0.05, ^{##}*p*<0.01). (B)
 581 Histological analysis of pulmonary arteries showing mean vessel diameter and media
 582 thickness normalised to vessel diameter in arteries with diameter in groups of 25-50 µm and
 583 51-100 µm, respectively. *n*= 4-8/group. Representative example of an untreated (MCT –) and
 584 treated (MCT +) *Trpm4*^{+/+} animal is shown above. Differences between groups were
 585 evaluated by ANOVA test ([#]*p*<0.05, ^{##}*p*<0.01). (C) Echocardiographic parameters assessing
 586 pulmonary arterial pressure. Representative example of PAAT (left panel) and RVS' (right
 587 panel) in an untreated (MCT –) and treated (MCT +) *Trpm4*^{+/+} animal is shown above the
 588 data table. *n*= 10-15/group. Differences between groups were evaluated by ANOVA test
 589 (comparing to untreated: [#]*p*<0.05, ^{##}*p*<0.01). CL cycle length, MCT Monocrotaline, *n*
 590 number, PAAT pulmonary arterial acceleration time, RVS' velocity of the maximal tricuspid
 591 anular systolic motion, *Trpm4*^{-/-} TRPM4-deficient, *Trpm4*^{+/+} wild type.

592
 593 **Figure 4: Right ventricular contractile function of TRPM4-deficient rats is comparable**
 594 **to wild type.** Contractility measurements of isolated intact right ventricular papillary muscles
 595 (A-D): twitch force of papillary muscles under basal conditions (A). Time to peak, relaxation₅₀
 596 time and twitch velocity (data table, B). Twitch force in relation to pacing-frequency (C) and
 597 after application of Isoprenaline (D). *n*= 5-11/group. Differences between groups were
 598 evaluated by ANOVA test (comparing to untreated [#]*p*<0.05, ^{##}*p*<0.01). (E) Echocardiographic
 599 parameters evaluating right ventricular contractile function in untreated rats (left panel) and
 600 42 days after Monocrotaline injection (right panel). Representative example of TAPSE in an
 601 untreated (MCT –) and treated (MCT +) *Trpm4*^{+/+} animal is shown on the left. *n*= 10-
 602 15/group. Differences between groups were evaluated by ANOVA test (comparing to
 603 untreated: [#]*p*<0.05, ^{##}*p*<0.01). FS fractional shortening, MCT Monocrotaline, *n* number,
 604 RVFAC right ventricular fractional area change, TAPSE tricuspid annular plane systolic
 605 excursion, RV D' velocity of the maximal tricuspid anular diastolic motion, *Trpm4*^{-/-} TRPM4-
 606 deficient, *Trpm4*^{+/+} wild type.

607
 608 **Figure 5: Decreased expression of TRPM4 in right ventricular myocardium of wild type**
 609 **rats under right ventricular pressure load.** Western Blot of right and left ventricular
 610 myocardium of untreated *Trpm4*^{+/+} rats and 42 days after Monocrotaline injection. (A)

611 Western blot of right ventricular myocardium in untreated and treated animals (left).
 612 Quantitative statistical analysis of TRPM4 protein-expression normalised to Calnexin-
 613 expression (right). TRPM4-deficient tissue serves as negative control. n=4-6/group.
 614 Differences between groups were evaluated by t-test (**p<0.01 (B) Western blot of left
 615 ventricular myocardium in untreated and treated animals (left). Quantitative statistical
 616 analysis of TRPM4 protein-expression normalised to Calnexin-expression (right). TRPM4-
 617 deficient tissue serves as negative control. n=4-6/group. MCT Monocrotaline, Trpm4^{-/-}
 618 TRPM4-deficient, Trpm4^{+/+} wild type.

619

620 **10. Glossary**

621

622	Ao	aortic valve diameter
623	FS	fractional shortening
624	GAPDH	Glyceraldehyde-3-phosphat-Dehydrogenase
625	IVSs/ IVSd	interventricular septum systolic/diastolic
626	LV	left ventricle
627	LVAWs/ LVAWd	left ventricular anterior wall systolic/diastolic
628	LVEDS/ LVEDD	left ventricular endsystolic/enddiastolic diameter
629	LVPWd/LVPWs	left ventricular posterior wall systolic/diastolic
630	MCT	Monocrotaline
631	RV	right ventricle
632	RVFAC	Right ventricular fractional area change
633	RVA ES/ RVA ED	Right ventricular area systolic/diastolic
634	RVAWOTs/ RVAWOTd	right ventricular anterior wall at outflow tract syst./diast.
635	RVAWs /RVAWd	right ventricular anterior wall systolic/diastolic
636	RVDs / RVDd	right ventricular diameter systolic/diastolic
637	RVD'	velocity of the maximal tricuspid anular diastolic motion
638	RVOTs / RVOTd	right outflow tract dimension systolic/diastolic
639	RVS'	velocity of the maximal tricuspid anular systolic motion
640	SF	shortening fraction
641	SV	stroke volume
642	TAPSE	tricuspid annular plane systolic excursion
643	TRPM4	transient receptor potential melastatin 4
644	Trpm4 ^{-/-}	TRPM4-deficient
645	Trpm4 ^{+/+}	wild type

Figure 1

Figure 1

A

Figure 3

Figure 3

Figure 4

Figure 4

Figure 5

Figure 5

Supplementary material to:

TRPM4 modulates right ventricular remodelling under pressure load accompanied with decreased expression level

Short title: Right ventricular hypertrophy is modulated by TRPM4

Wiebke Frede^{1,2}, Rebekka Medert², Tanja Poth³, Matthias Gorenflo¹, Rudi Vennekens⁴,
Marc Freichel², Sebastian Uhl^{1,2}

¹ Department Pediatric and Congenital Cardiology, University Medical Center, Heidelberg, Germany

² Institute of Pharmacology, University Medical Center, Heidelberg, Germany

³ Center for Model System and Comparative Pathology, University Medical Center, Heidelberg, Germany

⁴ Laboratory of Ion Channel Research, Department of Cellular and Molecular Medicine,
KULeuven, Leuven, Vlaams Brabant, Belgium

Corresponding author:

Sebastian Uhl, Institute of Pharmacology, University Medical Center, Heidelberg, Germany

phone: 0049/6221-54-86861, fax: 0049/6221-54-8644

email: sebastian.uhl@med.uni-heidelberg.de

A

B

C

	untreated		Monocrotaline	
	Trpm4 ^{+/+}	Trpm4 ^{-/-}	Trpm4 ^{+/+}	Trpm4 ^{-/-}
LVAWs (mm)	3.35±0.10	3.08±0.10	3.15±0.14	3.19±0.11
LVAWd (mm)	1.86±0.10	1.77±0.07	1.96±0.10	2.1±0.14
IVSs (mm)	3.17±0.15	3.42±0.17	3.32±0.14	3.87±0.12
LVEDS (mm)	4.43±0.24	4.74±0.26	4.61±0.31	4.72±0.12
LVPWs (mm)	3.14±0.09	3.16±0.17	2.98±0.14	2.89±0.22
IVSd (mm)	1.19±0.06	1.88±0.06	1.90±0.17	2.24±0.13
LVEDD (mm)	7.79±0.14	8.47±0.17	7.98±0.24	8.30±0.27
LVPWd (mm)	2.07±0.12	2.19±0.10	2.15±0.13	1.74±0.09 [#]
EF (%)	71±3.1	66±4.3	62±2.5	62±2.4
FS (%)	44±1.7	44±2.2	42±3.1	42±2.9
SV [μl]	375±31	448±46	352±21	427±0.26

[#] p<0.05 vs untreated

Supplementary figure 1: Monocrotaline-injection does not induce left ventricular hypertrophy or changes in left ventricular function in wild type and TRPM4-deficient rats. Weight of left ventricle plus interventricular septal wall in absolute (A) and in relation to tibia length (B). n=10-12/group. Differences between groups were evaluated by ANOVA test. (C) Echocardiographic parameters for left ventricular dimension and pump function. n=10-15/group. Differences between groups were evaluated by ANOVA test (comparing to untreated: [#]p<0.05). AW anterior wall, (e)d (end)diastolic, (e)s (end)systolic, FS fractional shortening, IVS interventricular septum, LV left ventricle, MCT Monocrotaline, PW posterior wall, SV stroke volume, Trpm4^{-/-} TRPM4-deficient, Trpm4^{+/+} wild type.

A

B

C

D

Supplementary figure 2: Data acquisition of contractile function of isolated right ventricular papillary muscles in rat and alterations of frequency-dependent and Isoprenaline-mediated twitch force of wild type and TRPM4-deficient rats after Monocrotaline-treatment. (A) Single twitch of a papillary muscle with analysed parameters: twitch force, time to peak (TTP) and halfmaximal relaxation time (R_{50}). (B) Representative traces of single twitches of wild type and TRPM4-deficient rats in untreated animals and after Monocrotaline-treatment. (C) Twitch force (force-frequency relation) under different pacing frequencies normalised to twitch force at 0.5 Hz. (D) Twitch force after Isoprenaline-application normalised to basal twitch force (B). $n=5-11/\text{group}$. Differences between groups were evaluated by ANOVA test (comparing to untreated: ## $p<0.01$). Trpm4-/- TRPM4-deficient, Trpm4+/+ wild type.