ONLINE DATA SUPPLEMENT

Supplemental Material and Methods

MCT-model

Male Wistar rats were used (n=23; weight, 125 to 150 g; Harlan, Horst, the Netherlands). PH was induced by a single subcutaneous injection of MCT, 60 mg/kg body mass; monocrotaline, Sigma-Aldrich, Zwijndrecht, the Netherlands) dissolved in sterile saline. Control group received a saline injection.(1) Two weeks after MCT injection, PH status was confirmed by echocardiography and animals were randomized into three groups: Controls (n=5); MCT-sham (n=9) or MCT-RD rats (n=9). At end-of-study (week 6, or when animals manifest signs of right heart failure), echocardiography and RV catheterization with pressure-volume analyses were performed as described before (Figure S1A).(2,3)

Sugen hypoxia-model

Male Sprague Dawley rats were used (n=26; weight, 125 to 150 g; Charles River, Sulzfeld, Germany). PH was induced by a single subcutaneous injection of vascular endothelial growth factor inhibitor (SU5416, 25 mg/kg body mass; Tocris Bioscience, Bristol, UK) dissolved in 0.5% carboxymethylcellulose followed by 4 weeks of hypoxia (10% oxygen; Biospherix Ltd, New York, NY, USA) maintained by a nitrogen generator (Avilo, Dirksland, The Netherlands) and re-exposed to normoxia for maximum 10 weeks. Control group received a carboxymethylcellulose injection and was exposed to normoxia until the end of the protocol.(4) At week 6, PH status was confirmed by echocardiography and animals were randomized into three groups: Controls (n=6); SuHx-sham (n=10) or SuHx-RD rats (n=10). At end-of-study (week 10, or when animals manifest signs of right heart failure),

echocardiography and RV catheterization with pressure-volume analyses were performed as described before (Supplement, Figure S1B).(2,3)

Hemodynamic evaluation

Echocardiography

Rats were evaluated by echocardiography prior to renal denervation and at the end of study. Transthoracic echocardiographic measurements (ProSound SSD-4000 system equipped with a 13-MHz linear transducer UST-5542, Aloka, Tokyo, Japan) were performed under anesthetized and spontaneously breathing rats (isoflurane 2.0% in 1:1 O2/air mix; Pharmachemie, Haarlem, The Netherlands).(1) Analyses were performed off-line (Image-Arena 2.9.1, TomTec Imaging Systems, Unterschleissheim, Munich, Germany). Parameters for right ventricular (RV) function were: cardiac output (Doppler-derived stroke volume, heart rate), and tricuspid annular plane systolic excursion (TAPSE). Parameters for RV remodelling were: RV end diastolic diameter (RVEDD) and RV wall thickness. Total pulmonary vascular resistance (tPVR) was estimated by Poiseuille's law.(6-8)

Right ventricle catheterization and systemic blood pressure measurement

At the end of study rats were anesthetized with isoflurane (induction: 4.0% in 1:1 O2/air mix; maintenance: 2.0% in 1:1 O2/air mix), intubated (16G Teflon tube) and attached to a mechanical ventilator (Micro-Ventilator, UNO, Zevenaar, The Netherlands; ventilator settings: breathing frequency 75/min, pressures 9/0 cmH_2O, inspiratory/expiratory ratio 1:1). Rats were placed on a warming pad to maintain body temperature, and a carotid artery

catheterization (for systemic blood pressure measurement) followed by open-chest RV catheterization was performed. For the RV catheterization, the thorax was opened and the heart exposed, a temporary suture was placed around the inferior vena cava and a (23G needle) puncture in the right ventricle was performed. A combined pressure-volume catheter (SPR-869, Millar Instruments, Houston TX) was inserted into the right ventricle and positioned in the long axis. The signals (processed by MPVS-300, Millar Instruments), obtained at steady state (at least 10s) and during temporary vena cava occlusion were digitally recorded (2.0 kHz sampling rate; Chart 5.5.6, ADInstruments, Sydney, Australia) and analyzed off-line, using (LabChart 8, ADInstruments, Sydney, Australia) with custom-made algorithms (programmed in MATLAB R2007b, The MathWorks, Natick MA). Stroke volume (in RVU) derived from the conductance catheter was calibrated using the stroke volume (in ml) from echocardiogram as reference.(3)

Histomorphometric analyses of heart and lungs

After the hemodynamic evaluation, rats were euthanized (by exsanguination under isoflurane), and heart, lungs and other major organs were harvested. Lungs were weighted; left lobe was filled by 1:1 mix of saline and cryofixative (Tissue-Tek O.C.T. compound, Sakura, Fintek, Europe, Zoeterwolde, The Netherlands), and snapfrozen in liquid nitrogen. The middle right lobe was used to measured wet/dry lung mass ratio. The heart was perfused, weighted, dissected and snap-frozen in liquid nitrogen. Images were collected using a Motic microscope (BA210, Wetzlar, Germany) and a digital tablet camera (VisiCam ® TC10, VWR International B.V, Amsterdam, The Netherlands). ImageJ for Windows 1.48 software

(National Institutes of Health, USA) was used for image analysis, taking the pixel-to-aspect ratio into account.

Immunofluorescence

Frozen lung sections were kept for 30 min at room temperature and then immersed in a Coplin jar containing phosphate buffered saline (PBS) with 0.1% Tween 20 (PBST). Sections were fixed with 4% paraformaldehyde/PBS for 10 min. Sections were incubated in 0.3% Triton for 5 min, blocked with 5% bovine serum albumin (BSA) in PBS for 15 min, and incubated with primary antibodies in a humid chamber overnight at room temperature (1:50 for AT1-receptor, 1:100 for SM22; Santa Cruz); MR (1:200, kindly provided by Prof Marc Lombès, INSERM UMR-S 1185/Univ. Paris-Sud, Faculté de Médecine, Université Paris-Saclay, Le Kremlin Bicêtre, France), and Ki67 (1:100, Chemicon). Anti α-smooth muscle actin- Cy3 antibody (1:200; Sigma) was incubated for 1 hour at room temperature. After washing in PBST (3×5 min) the samples were incubated with appropriate secondary antibodies conjugated to either FluoProbes 547 anti-rabbit, FluoProbes 647 anti-goat and anti-rabbit (Invitrogen, Massachusetts, USA) for 2 h, washed in PBST (2×10 min), rinsed in PBS (5 min) and mounted in Prolong gold with DAPI (Invitrogen, Massachusetts, USA). Images were taken using LSM700 confocal microscope (Zeiss, Marly Le Roi, France).

Quantification of proliferation activity

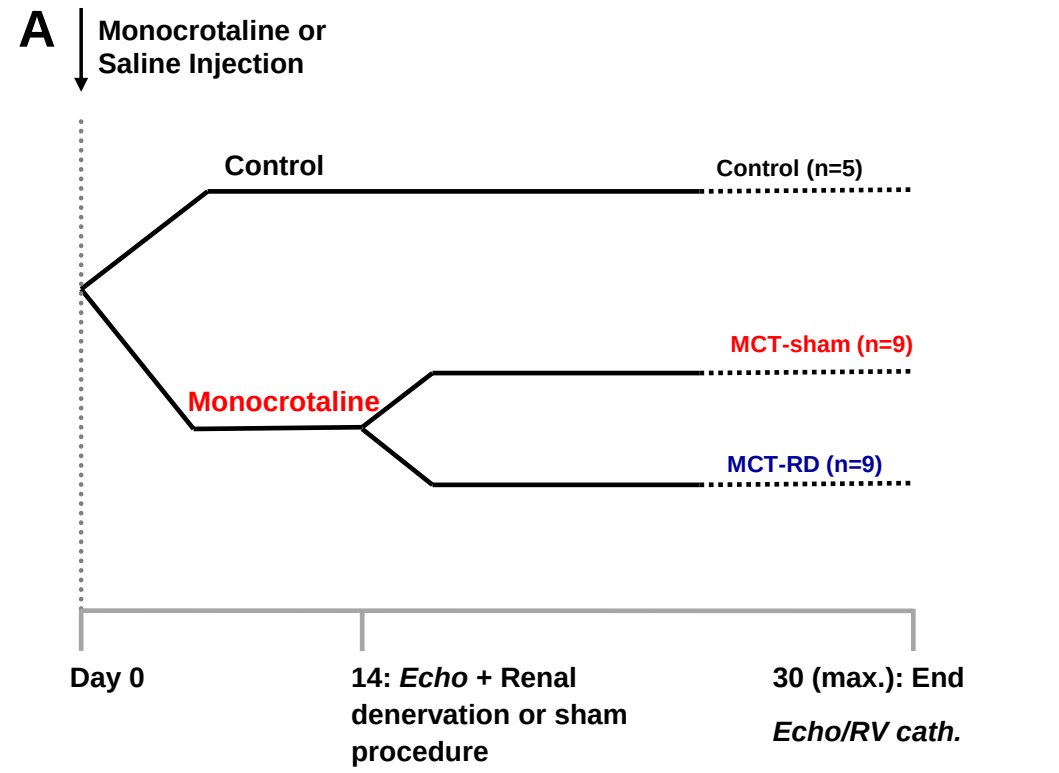
A minimum of 20 randomly selected vessels per sample were quantified by counting positive cells with a positive (Ki67) signal per total area.

Protein expression by Western Blot

RV tissue was homogenized in RIPA buffer containing phosphatase and protease inhibitors (Sigma-Aldrich, St. Louis, USA). The protein concentration was quantify by the Pierce 660nm protein assay kit (Thermo Scientific, Pierce Biotechnology, Rockford, USA). 30µg of protein was used to detect the expression of angiotensin II type 1 receptor (AT1-receptor monoclonal anti-mouse; 1:1000; Ab9391, Abcam, Cambridge, UK) and mineralocorticoid receptor (MR monoclonal anti-rabbit MR-39N; 1:1000; courtesy of Prof Marc Lombès, INSERM UMR-S 1185/Univ. Paris-Sud, Faculté de Médecine, Université Paris-Saclay, Le Kremlin Bicêtre, France). The protein amount was normalized by the loading control, GAPDH (1:50000; G9295, Sigma-Aldrich, St. Louis, USA).

Figure S1 - Study design

A: Monocrotaline as PH experimental model: Study design of monocrotaline as PH experimental model. Control: n=5, MCT-sham: n=9, MCT-RD: n=9; Day 0: Monocrotaline (60 mg/kg, s.c.) or saline injection. Day 14: Echocardiogram (Echo) followed by renal denervation or sham procedure. At the end of study, maximum day 30, or when animals develop signs of heart failure, echocardiogram and right ventricle (RV) catheterization (cath.) were performed. **B:** SU5416 combined with chronic hypoxia: Study design of SU5416 combined with chronic hypoxia (SuHx), as PH experimental model. Control: n=6, SuHx-sham: n=10, SuHx-RD: n=10; Week 0: SU5416 (25 mg/kg, s.c.) or 0.5% carboxymethylcellulose (CMC) injection and placed in hypoxia (10% oxygen) for 4 weeks. At week 6 (normoxia), echocardiogram (Echo) followed by renal denervation (RD) or sham procedure. At the end of study, at week 10 or when animals develop signs of heart failure, echocardiogram and right ventricle (RV) catheterization (cath.) were performed.


A
Monocrotaline or
Saline Injection
Control
Control (n=5)
MCT-sham (n=9)
Monocrotaline
MCT-RD (n=9)
Day 0
14: Echo + Renal denervation or sham procedure
30 (max.): End
Echo/RV cath.

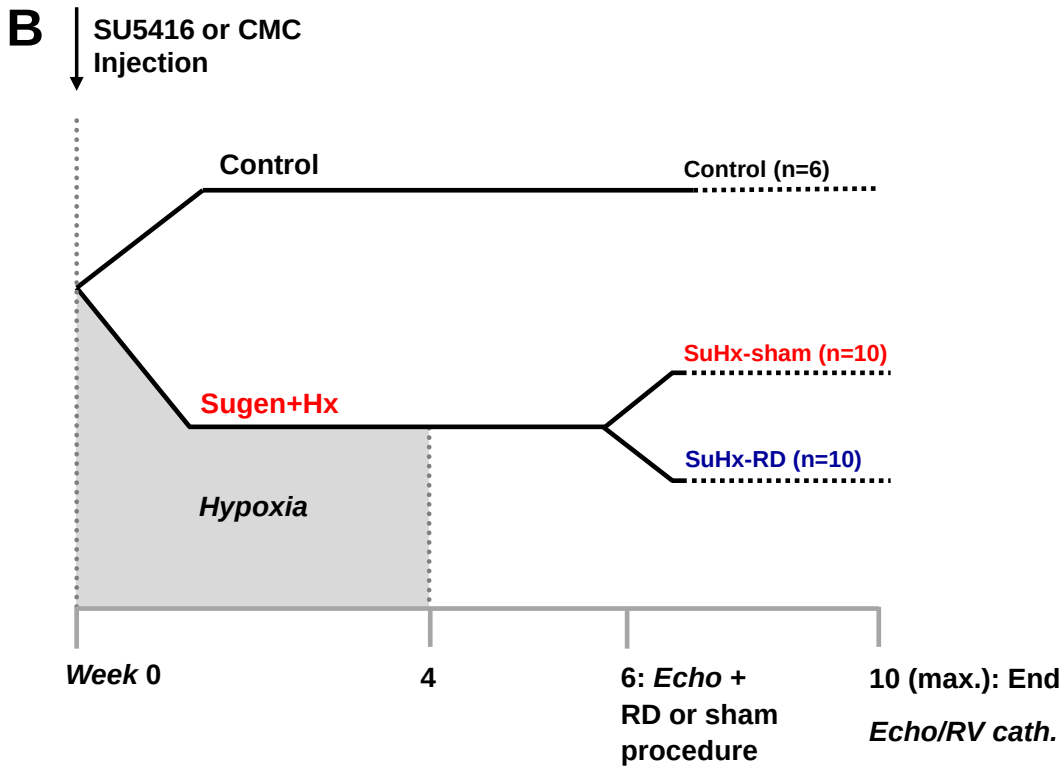
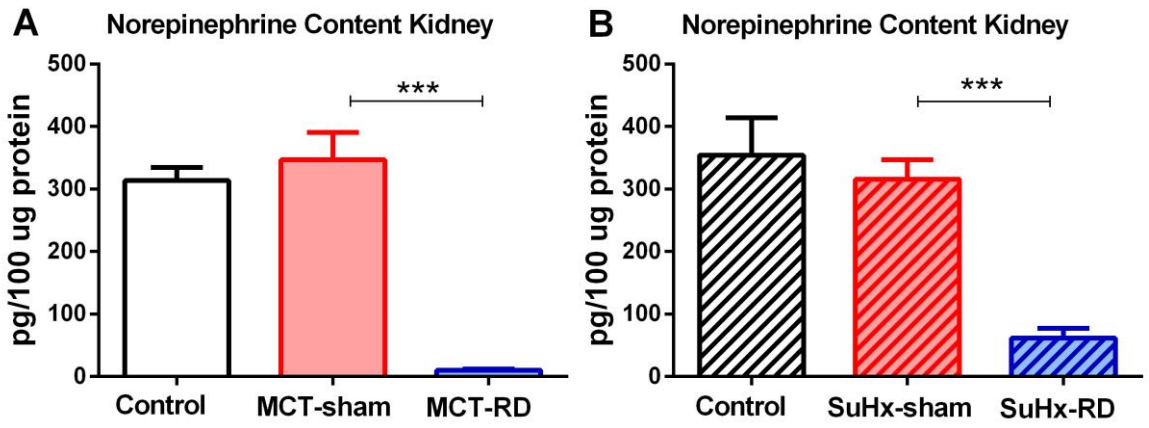

B
SU5416 or CMC
Injection
Control
Control (n=6)
SuHx-sham (n=10)
Sugen+Hx
SuHx-RD (n=10)
Hypoxia
Week 0
4
6: Echo +
RD or sham
procedure
10 (max.): End
Echo/RV cath.

Figure S2 - Norepinephrine content in kidney and aldosterone plasma levels

The renal denervation procedure was successfully performed, indicated by a significant reduction of the norepinephrine content in kidney tissue (**A:** monocrotaline model; MCT and **B:** sugen+hypoxia model; SuHx) in both renal denervated (RD) groups (blue bars) in comparison to sham (red bars). **C-D:** Aldosterone plasma levels were not changed after RD. On right side: Control: n=5, MCT-sham: n=9, MCT-RD: n=9; On left side: Control: n=6, SuHx-sham: n=10 and SuHx-RD: n=10. Data presented as mean ± SEM. . One-way ANOVA followed by Bonferroni correction; ***p<0.001 versus RD.



	Control	MCT-sham)	MCT-RD	p-value	
	(n=5)	(n=9	(n=9)	Control vs. MCT	MCT vs. RD
Body mass (g)	409±21	277±10	285±5	<0.001	>0.999
Tibia length (mm)	35.2±0.2	33.3±0.4	33.8±0.3	0.006	>0.999
Lungs/tl (g/mm*1000)	44.2±1.2	77.1±3.9	73.2±2.3	<0.001	>0.999
RV mass/tl (g/mm*1000)	7.2±0.7	12.0±0.6	10.3±0.3	<0.001	0.03
LV + S mass/tl (g/mm*1000)	24.1±0.8	19.1±1.1	17.5±0.7	0.008	0.58
RV/(LV+S)	0.3±0.02	0.6±0.02	0.6±0.02	<0.001	0.86
LV- CSA (μm^2)	464±15	377±21	347±16	<0.001	>0.05
LV fibrosis (%)	0.54±0.04	0.64±0.05	0.62±0.06	>0.05	>0.05
Liver/tl (g/mm*1000)	494±39	329±23	337±35	<0.001	>0.999
Left Kidney/tl (g/mm*1000)	39.3±1.8	29.8±1.1	30.8±0.7	<0.001	>0.999
Right kidney/tl (g/mm*1000)	39.8±1.3	30.6±0.9	30.7±0.6	<0.001	>0.999

	Control	SuHx-sham	SuHx-RD	p-value

Table S1: Autopsy data, monocrotaline (MCT) model. All data are presented as mean ± SEM One-way ANOVA followed by Bonferroni correction; Abbreviations: RD= renal denervation; tl= tibia length; RV=right ventricle; LV + S=left ventricle (including septum); RV/(LV+S)= RV over LV (including septum) mass ratio; LV-CSA= left ventricle cross sectional area.

	(n=6)	(n=10)	(n=10)	Control vs. SuHx	Sham vs. RD
Body mass (g)	546±26	448±18	454±10	0.002	>0.999
Tibia length (mm)	42.2±0.3	39.5±0.4	40.1±0.3	<0.001	0.17
Lungs/tl (g/mm*1000)	38.6±1.7	54.9±1.9	52.9±1.5	<0.001	0.85
RV mass/tl (g/mm*1000)	6.0±0.4	15.35±1.2	10.3±0.3	<0.001	0.44
LV + S mass/tl (g/mm*1000)	17.3±1.2	23.3±1.3	20.9±0.9	0.002	0.14
RV/(LV+S)	0.3±0.02	0.67±0.05	0.67±0.04	<0.001	>0.999
LV- CSA (μm^2)	465±19	487±25	482±29	>0.05	>0.05
LV fibrosis (%)	0.17±0.02	0.34±0.04	0.31±0.02	<0.001	>0.05
Liver/tl (g/mm*1000)	400±21	400±19	376±13	>0.999	0.96
Left Kidney/tl (g/mm*1000)	36.7±2.1	36.3±1.3	36.9±1.1	>0.999	>0.999
Right kidney/tl (g/mm*1000)	38.3±2.3	36.6±1.2	37.7±1.2	>0.999	>0.999

Table S2: Autopsy data, sugen+hypoxia (SuHx) model. All data are presented as mean ± SEM. One-way ANOVA followed by Bonferroni correction; Abbreviations: RD= renal denervation; tl= tibia length; RV=right ventricle; LV + S=left ventricle (including septum); RV/(LV+S)= RV over LV (including septum) mass ratio.

	Control	MCT-sham	MCT-RD	p-value	
	(n=5)	(n=9)	(n=9)	Control vs. MCT-sham	Sham vs. RD
Cardiac output (mL/min)	116±15.7	76±9.6	91±8.3	0.04	0.76
Stroke volume (mL)	0.28±0.03	0.19±0.02	0.24±0.02	0.02	0.07
Heart rate (bpm)	405±7.8	399±11.0	356±11.0	0.99	0.006
TAPSE (mm)	3.8±0.19	3.1±0.06	3.2±0.19	0.01	0.56
RV wall thickness (mm)	0.46±0.03	0.63±0.05	0.63±0.03	0.04	0.94
RVEDD (mm)	2.8±0.14	3.5±0.14	3.7±0.15	0.01	0.50
PAAT/cl ($^{x}100$)	13.1±0.6	7.2±0.5	7.7±0.3	<0.001	0.43
eRVSP (mmHg)	33.8±2.2	64.9±3.4	61.3±2.2	<0.001	0.36
tPVR (mmHg/ml/min)	0.22±0.04	0.63±0.09	0.47±0.05	0.002	0.08

Table S3: Baseline echocardiography characteristics from monocrotaline (MCT) model. All data are presented as mean ± SEM; One-way ANOVA followed by Bonferroni correction; Abbreviations: RD= renal denervation; TAPSE= tricuspid annular plane systolic excursion; RVEDD= right ventricular end-diastolic diameter; PAAT/cl= pulmonary acceleration time divided by cycle length; eRVSP= estimated right ventricular systolic pressure; tPVR= total pulmonary vascular resistance.

	Control	SuHx-sham	SuHx-RD	p-value	
	(n=6)	(n=10)	(n=10)	Control vs. SuHx	Sham vs. RD
Cardiac output (mL/min)	82±3.5	53±1.2	58±3.9	<0.001	0.34
Stroke volume (mL)	0.24±0.01	0.17±0.003	0.19±0.01	0.002	0.22
Heart rate (bpm)	339±9.1	305±6.6	292±8.7	0.02	0.24
TAPSE (mm)	3.4±0.1	1.9±0.1	1.9±0.07	<0.001	0.64
RV wall thickness (mm)	0.82±0.05	1.5±0.05	1.6±0.09	<0.001	0.23
RVEDD (mm)	3.9±0.24	5.1±0.38	5.5±0.32	0.07	0.34
PAAT/cl (^{x}100)	12.6±0.4	5.1±0.3	5.1±0.3	<0.001	>0.999
eRVSP (mmHg)	35.7±1.5	81.7±2.5	81.8±2.6	<0.001	0.98
tPVR (mmHg/ml/min)	0.29±0.02	0.99±0.04	0.87±0.04	<0.001	0.09

Table S4: Baseline echocardiography characteristics from sugen+hypoxia (SuHx) model. All data are presented as mean ± SEM; One-way ANOVA followed by Bonferroni correction; Abbreviations: RD= renal denervation; TAPSE= tricuspid annular plane systolic excursion; RVEDD= right ventricular end-diastolic diameter; PAAT/cl= pulmonary acceleration time divided by cycle length; eRVSP= estimated right ventricular systolic pressure; tPVR= total pulmonary vascular resistance**.**

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