Urocortin-2 improves right ventricular function and attenuates pulmonary arterial hypertension

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Received 28 June 2017; revised 26 December 2017; editorial decision 20 March 2018; accepted 22 March 2018; online publish-ahead-of-print 23 March 2018

Time for primary review: 55 days

Aims

Pulmonary arterial hypertension (PAH) is a devastating disease and treatment options are limited. Urocortin-2 (Ucn-2) has shown promising therapeutic effects in experimental and clinical left ventricular heart failure (HF). Our aim was to analyse the expression of Ucn-2 in human and experimental PAH, and to investigate the effects of human Ucn-2 (hUcn-2) administration in rats with monocrotaline (MCT)-induced pulmonary hypertension (PH).

Methods and results

Tissue samples were collected from patients with and without PAH and from rats with MCT-induced PH. hUcn-2 (5 µg/kg, bi-daily, i.p., for 10 days) or vehicle was administered to male wistar rats subjected to MCT injection or to pulmonary artery banding (PAB) to induce right ventricular (RV) overload without PAH. Expression of Ucn-2 and its receptor was increased in the RV of patients and rats with PAH. hUcn-2 treatment reduced PAH in MCT rats, resulting in decreased morbidity, improved exercise capacity and attenuated pulmonary arterial and RV remodelling and dysfunction. Additionally, RV gene expression of hypertrophy and failure signalling pathways were attenuated. hUcn-2 treatment also attenuated PAB-induced RV hypertrophy.

Conclusions

Ucn-2 levels are altered in human and experimental PAH. hUcn-2 treatment attenuates PAH and RV dysfunction in MCT-induced PH, has direct anti-remodelling effects on the pressure-overloaded RV, and improves pulmonary vascular function.

Keywords

Urocortin-2 • Pulmonary hypertension • Vascular remodelling • Cardiac hypertrophy • Right ventricular failure

1. Introduction

Pulmonary arterial hypertension (PAH) is a rare and severe condition characterized by intense pulmonary vascular remodelling, vasoconstriction, endothelial dysfunction, inflammation and *in situ* thrombosis, leading to elevated pulmonary vascular resistance (PVR) and right ventricular (RV) failure.^{1,2} Available therapies decrease patients' risk of short-term mortality and clinical worsening³; however, long-term prognosis remains poor.⁴ RV function is the main predictor of the outcome in PAH⁵ and

thus new therapies should also protect against RV maladaptation and failure.⁶

Urocortin-2 (Ucn-2) is an endogenous vasoactive peptide of the corticotropin-releasing hormone (CRH) family that binds with high affinity to the Type 2 CRH receptor (CRHR2), which is expressed abundantly in the cardiovascular system.⁷ Ucn-2 promotes important cardiac haemodynamic effects, including vasodilation and positive cardiac inotropic, chronotropic, and lusitropic actions.⁸ Additionally, the Ucn-2/CRHR2 system exhibits cardioprotective actions, preventing cell

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necrosis and apoptosis^{9,10} and decreasing infarct size in the rat heart after ischaemia-reperfusion injury.^{10–12}

Acute Ucn-2 administration in experimental heart failure (HF) has beneficial haemodynamic effects, while suppressing adverse neurohormonal responses,^{13,14} whereas chronic treatment after experimental myocardial infarction improves cardiac function and structure.¹⁵ In patients with stable congestive¹⁶ and acute decompensated HF,¹⁷ intravenous Ucn-2 produces an increase in cardiac output (CO) and left ventricular (LV) ejection fraction (EF) in association with a reduction in systemic vascular resistance, blood pressure and cardiac work. Plasma levels of Ucn-2 are elevated in patients with abdominal aortic aneurysm,¹⁸ LV systolic dysfunction with coronary artery disease¹⁹ and non-ischaemic dilated cardiomyopathy,²⁰ suggesting a potential role of Ucn-2 as a biomarker in heart disease. Additionally, plasma concentrations of N-terminal-proUcn-2 are independently and inversely related to 2-year survival in HF.²¹

Ucn-2 is reported to induce vasorelaxation of human coronary arteries independently of the endothelium,²² while in healthy volunteers, arterial vasodilatation appears to be at least partly mediated by endothelial nitric oxide.²³ Ucn-2 is also associated with abdominal aortic aneurysm and mediates anti-proliferative effects on vascular smooth muscle cells.¹⁸ Moreover, Ucn-2 has sustained blood pressure-lowering effects in hypertensive rats and attenuates hypertension-induced LV hypertrophy and dysfunction,²⁴ suggesting a novel therapeutic approach for systemic arterial hypertension.

Considering the need for new cardiac-directed drugs in PAH, and the cardioprotective effects of Ucn-2 in HF, we hypothesized that Ucn-2 might have a potential role in the treatment of established PAH. In this study, we evaluated the expression of Ucn-2/CRHR2 signalling in human and experimental PAH, and investigated the functional and structural effects of chronic human Ucn-2 (hUcn-2) treatment on the heart and pulmonary vessels of rats with monocrotaline (MCT)-induced pulmonary hypertension (PH). To distinguish cardiac-specific actions from effects on the pulmonary vasculature, hUcn-2 treatment was also studied in an experimental model of pressure overload by pulmonary artery banding (PAB), which results in RV loading without PAH.

2. Methods

All patients or their legal representatives (in the case of autopsy) gave informed consent before the beginning of the study in accordance to the declaration of Helsinki, and institutional guidelines were approved by Institut Universitaire de Cardiologie et de Pneumologie de Québec (CER-20773). The usage of human tissues follow recommendations published,²⁵ in collaboration with the Institut Universitaire de Cardiologie et de Pneumologie de Québec site of the Respiratory Health Network tissue bank of the Fonds de recherche du Québec – Santé (FRQS) (www.tissuebank.ca). 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 (PTDC/DTP-FTO/0130/2012; 0421/000/000/2013). A detailed method section is available in [Supplementary material online](#).

2.1 Human samples

Human RV samples were collected from patients with and without PAH (see [Supplementary material online, Table S1](#)) at the time of cardiac surgery, heart transplantation or autopsy (within 3 h of death). Samples were categorized according to clinical history and designated as compensated RV (CRV) or RV failure (RVF).²⁶ Blood samples were also collected from PAH patients and age-matched controls.²⁷

2.2 Animal study

PAH was induced as previously described.²⁸ Seven-week-old male Wistar rats (Charles River Laboratories, 180–200 g) randomly received an injection of MCT (60 mg/kg, s.c., Sigma-Aldrich) or an equal amount of vehicle. Two weeks after MCT/vehicle administration, animals were further randomly divided to receive hUcn-2 (Bachem) at a dose of 5 µg/kg (bi-daily, i.p.) or an equal amount of vehicle (0.9% NaCl) for 10 days, resulting in four groups: Ctrl + vehicle (C); Ctrl + hUcn-2 (CU); MCT + vehicle (M); MCT + hUcn-2 (MU). After the treatment period, animals were submitted to exercise testing and echocardiographic and invasive haemodynamic analysis, with subsequent sample collection for *in vitro* functional studies, morphological, histological, and molecular analysis, as detailed in [Supplementary material online](#). To determine whether MCT-induced PH was already present prior to therapy, a subgroup of animals underwent the same experimental protocol and was evaluated at an earlier time point (14 days) (see [Supplementary material online, Figures S7 and S8](#)).

An additional subgroup of rats was subjected to PAB (16-gauge) or sham procedure,²⁹ where applying a pulmonary artery constriction resulted in a degree of hypertrophy and RV overload identical to the MCT-induced PH model (see [Supplementary material online, Figure S6](#)). Two weeks after PAB, animals randomly received hUcn-2 or vehicle for 10 days, resulting in three groups: Sham + vehicle (S); PAB + vehicle (B), and PAB + hUcn-2 (BU). Sampling was performed as in the MCT protocol.

2.3 Survival analysis

The defined end-point for the survival analysis was a 20% loss of body weight plus oedema/ascites and decreased movement. When this end-point was reached, animals were euthanized with an anaesthetic overdose (sodium pentobarbital 100 mg/kg, Eutasil, CEVA). Only those animals reaching the end-point before evaluation at study-end were considered valid and used in the survival analysis.

2.4 Exercise capacity test

Animal exercise capacity was tested on a close-chamber treadmill set with electric stimulation (LE8710R, Columbus Instruments) coupled to a gas analyser. Peak effort to assess maximal oxygen consumption ($\text{VO}_{2\text{max}}$)³⁰ and endurance tests³¹ were evaluated in different sets of animals, and were performed 13 and 23 days after PAH induction.

2.5 Echocardiography

Echocardiographic analysis,²⁸ was performed 13 days (baseline) and 24 days after PAH induction. Animals were lightly anaesthetized (inhalation of 8% sevoflurane for induction, and 2–3.5% for maintenance). Echocardiographic evaluation was performed using a 15 MHz probe (GE Healthcare) and a Acuson Sequoia C512 echocardiography system (Siemens), for determination of pulmonary flow and RV structure.

2.6 Invasive haemodynamic evaluation

Following echocardiographic evaluation, the animals were intubated and ventilated. Using an open chest approach, as previously described in,²⁸ 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 anaesthetic overdose, blood samples were taken, the animals exsanguinated and heart and lungs collected *en bloc*.

2.7 Morphometric and histological analysis

Following removal, the lungs, RV and LV + septum (LV + S) were carefully separated and weighed. Tibia length was measured for weight normalization. RV and lung samples were collected, snap frozen in liquid nitrogen, and stored at -80°C. For mRNA quantification, samples were submerged in RNA stabilization reagent (RNAlater, Qiagen), and for histological analysis, samples were stored in buffered 10% formaldehyde.

2.8 Assessment of isolated pulmonary artery endothelial function and vascular acute analysis

As previously described in,²⁸ second-generation pulmonary arteries (200–400 µm diameter) were dissected from the left upper lobe of rats. Arterial rings were isolated and mounted in a bath myograph system (720 MO, DMT). Maximum tension development was assessed with 80 mM KCl solution. For the determination of the acute vasodilatory effects of hUcn-2, arterial rings were contracted with phenylephrine (10^{-5} M), and a dose response to hUcn-2 (10^{-9} to 5×10^{-7} M) or vehicle (ddH₂O) was performed. Maximal relaxation to hUcn-2 ($E_{\max}$) and the concentration of hUcn-2 required for 50% of the maximal response (EC_{50}) were calculated. Pulmonary artery endothelial function was assessed by attaining a dose–response curve to acetylcholine (10^{-9} to 10^{-5} M) after pre-contraction with phenylephrine (10^{-5} M). Maximal relaxation to acetylcholine and EC_{50} were calculated. Analysis was performed offline (LabChart 7 Pro, ADInstruments).

2.9 In vitro studies in isolated skinned cardiomyocytes

RV samples (stored at -80°C) 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 403 A, Aurora Scientific) and a length controller (model 315 C-I, Aurora Scientific). Cell length was digitally adjusted through custom-designed software (series 600 A digital controller, Aurora Scientific), and steady-state passive force measured at increasing sarcomere lengths (1.8–2.3 µm). Force measurements were normalized to each cardiomyocyte cross-sectional area (CSA) and a minimum of three cells per animal measured.²⁹

2.10 Quantitative RT-PCR, immunoblotting, and enzyme-linked immunosorbent assay

For human samples, total RNA was isolated from tissue using the RNeasy micro kit according to the manufacturer's instruction, and mRNA reverse transcribed and amplified using one-step kit TaqMan probes. qRT-PCR was performed with the ABI Prism 7900 Sequence Detector (Applied Biosystems), and 18 s used as a housekeeping gene. The relative expression level of Ucn-2 and CRHR2 was determined by the $2^{-\Delta Ct} \times 100$.

For rat samples, 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 results presented in arbitrary units and normalized for the control group. Primers used were designed in-house (see [Supplementary material online, Table S2](#)).

For immunoblotting experiments, human and rat RV total protein was extracted and separated in a 10% SDS–PAGE gel, electroblotted into nitrocellulose membrane, and probed for primary antibodies (see [Supplementary material online, Table S3](#)). After washing, the membrane was probed with secondary antibodies (Li-COR Biosciences) and visualized using an Odyssey scanner (Li-COR Biosciences). Human RV CRHR2 expression was quantified using a horse-radish peroxidase conjugate secondary antibody (Bio-Rad) and ChemiDoc system, and normalized for total protein content (Amido Black, Sigma Aldrich).

Blood collected from rats and humans was centrifuged and dispensed plasma assayed for Ucn-2 concentrations using solid-phase sandwich enzyme-linked immunosorbent assay according to manufacturer's instructions (SEC585Ra and SEC585Hu, Cloud-Clone Corp.).

2.11 Statistical analysis

The results are presented as the mean $\pm$ S.E.M. Survival analysis was performed in R (version 3.4.1) using PwrGSD package (version 2.0), whereas GraphPad Software v7 (GraphPad Software Inc., San Diego, CA, USA) was used in all other parameters. 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. Where appropriate, Student *t*-test or one-way ANOVA with Tukey's method for post hoc comparisons were used. Survival analysis was estimated according to the Kaplan-Meier method and comparisons were performed using a generalized Fleming-Harrington test ($p = 1$, $q = 1$) for non-proportional hazards, which gives more weight to later events. A *P*-value of <0.05 was considered to be statistically significant.

3. Results

3.1 Levels of Ucn-2 and CRHR2 are altered in PAH-induced RVF and are modulated by chronic hUcn-2 treatment

Plasma concentrations of Ucn-2 were unchanged in human PAH compared with controls (*Figure 1A*). Measurement of Ucn-2 mRNA levels in the RV of PAH patients with RVF showed increased expression compared with non-failure patients (*Figure 1B*), whereas CRHR2 protein levels were found to be elevated in human PAH (*Figure 1* and *D*). There were no differences between male and female patients on both

Figure 1 Levels of Ucn-2 and CRHR2 are altered in PAH-induced RVF and are modulated by chronic hUcn-2 treatment. (A) Plasma Ucn-2 concentrations in patients with PAH ($n = 10\text{--}14/\text{group}$). (B and C) mRNA expression of Ucn-2 and CRHR2 protein levels in the RV of patients with CRV and RVF. (D) Representative blots of CRHR2 immunoreactivity in human RV samples ($n = 8/\text{group}$). (E) Plasma Ucn-2 concentrations in control and MCT-injected rats with (CU/MU) and without (C/M) hUcn-2 treatment ($n = 6\text{--}7/\text{group}$). (F and G) mRNA expression of Ucn-2 and CRHR2 in the RV of MCT-induced PH protocol ($n = 6\text{--}7/\text{group}$). (H and I) CRHR2 protein levels in the RV of MCT-induced PH protocol and corresponding representative blots of CRHR2 immunoreactivity ($n = 5\text{--}9/\text{group}$). (J and K) mRNA expression of Ucn-2 and CRHR2 in the lungs of MCT-induced PH protocol ($n = 9\text{--}13/\text{group}$). (L and M) CRHR2 protein levels in the lungs of MCT-induced PH protocol and corresponding representative blots of CRHR2 immunoreactivity ($n = 4/\text{group}$). Results are presented as the mean $\pm$ S.E.M. * $P < 0.05$ vs. CRV in (panels B and C). * $P < 0.05$, ** $P < 0.01$ vs. C; # $P < 0.05$ vs. M in (E–H). Student t-test was performed to compare groups in panels (A–C), while two-way ANOVA was used for all the other parameters presented.

plasma and RV mRNA Ucn-2 (see [Supplementary material online, Figure S1](#)). Plasma concentrations of Ucn-2 were increased in MCT-induced PH compared with controls, and hUcn-2 therapy in control and MCT rats did not chronically increase the Ucn-2 plasma concentrations (Figure 1E). MCT animals treated with hUcn-2 showed a reversal of RV Ucn-2 and CRHR2 mRNA expression to control levels compared with the MCT group without treatment, which were elevated (Figure 1F and G). Although there were no significant differences in MCT group, administration of hUcn-2 in control animals resulted in an increased CRHR2 protein expression (Figure 1H and I). Indeed, the CRHR2 receptor is present in cardiomyocytes from control and MCT RV (see [Supplementary material online, Figure S2](#)).

However, as seen for Ucn-2 in the lungs from PAH patients (see [Supplementary material online, Figure S1](#)), we did not find any differences between the groups in the expression of Ucn-2 and CRHR2 in experimental PAH (Figure 1J–M).

3.2 Treatment with hUcn-2 decreases morbidity and improved exercise capacity in experimental PAH

In our experimental protocol (Figure 2A) we observed that the mean bodyweight and gain of MCT-induced PH animals were significantly reduced compared with control animals after 24 days. Treatment with hUcn-2, while reducing bodyweight gain in

Figure 2 Effect of hUcn-2 on morbidity and exercise capacity. (A) Diagram of rat experimental MCT protocol. (B) Survival analysis in MCT-injected rats ($n = 47$ – 54 /group). (C and D) Mean body weight and bodyweight gain in Control and MCT-injected rats with (CU/MU) and without (C/M) hUcn-2 treatment ($n = 17$ – 54 /group). (E and F) Distance run and maximal oxygen consumption during endurance testing in all experimental groups ($n = 4$ – 7 /group). Results are presented as the mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. For panel (B), Kaplan-Meier survival analysis was performed. Two-way ANOVA repeated measures test were used for all the other parameters presented.

control animals, increased bodyweight gain in MCT animals (Figure 2C and D).

MU group showed a lower percentage of animals reaching the defined end-point, which serves as a surrogate for clinical worsening, and could potentially represent an improvement in late mortality (Figure 2B). Treatment with hUcn-2 for 10 days also significantly improved the exercise capacity, with increases seen in distance run and maximum rate of oxygen consumption (VO_{2max}) (Figure 2E and F).

3.3 hUcn-2 treatment reduces cardiac and pulmonary arterial remodelling in MCT-induced PH

In MCT animals, administration of hUcn-2 resulted in decreased RV and pulmonary vascular remodelling (Figure 3A). hUcn-2 treatment attenuated MCT-induced increases in both RV and right atrial dilation (Figure 3B and C) as well as the RV weight normalized to left ventricle plus septum weight (RV/LV + S) (Figure 3D). Lung weight normalized to tibia length (Lung/TL), was reduced by hUcn-2 treatment compared with the MCT group (Figure 3E). This shows a decrease of fluid build-up in the lungs, potentially as a result of improved LV diastolic function (see Supplementary material online, Figure S5) in treated animals. Animals with PAH and without

pharmacological intervention also presented increased cardiomyocyte CSA, as well as fibrosis deposition (Figure 3F and G). hUcn-2 treatment normalized both cardiomyocyte size and fibrotic tissue deposition. Pulmonary small artery (15–50 µm) remodelling was also decreased in MU group, as measured by a decline in mean lumen/total ratio and mean medial thickness (Figure 3H and I). A detailed morphometric analysis to evaluate vascular remodelling in all, small (15–50 µm) and medium (50–100 µm) pulmonary arterioles can be found in Supplementary material online, Figure S3.

3.4 hUcn-2 attenuates pulmonary endothelial dysfunction and induces vasodilatory effects in MCT-induced PH

Isolated pulmonary arterial rings pre-contracted with phenylephrine (10^{-5} M), exhibited a vasodilation response when incubated with hUcn-2 (10^{-9} to 5×10^{-7} M) (Figure 4A and B). In rings isolated from MCT animals, although present, vasodilation was significantly decreased, when compared with controls (Figure 4C). The vasodilation response to hUcn-2 in both groups was independent of the endothelium (see Supplementary material online, Figure S4). Furthermore, pre-treatment with hUcn-2 (50 nM) attenuated phenylephrine-induced vasoconstriction in pulmonary arterial

Figure 3 Treatment with hUcn-2 attenuates RV and pulmonary vascular remodelling. (A) Representative echocardiographs (first line PSAX = parasternal short axis view; second line A4C = apical four chamber view) of Control and MCT-injected rats with (CU/MU) and without (C/M) hUcn-2 treatment; inter-ventricular septum hypertrophy and bowing shown by white arrow heads, and RV dilation by white double arrow. Representative haematoxylin and eosin (H&E) and picosirius red staining of RV samples (third and fourth lines) and representative H&E staining of lung samples (fifth line) of all experimental groups. Scale bars represent 50 μ m (400 $\times$ magnification) in H&E stainings, and 25 μ m (200 $\times$ magnification) in picosirius red staining. (B and C) RAA (right atrial area) and RVEDD analysis ($n = 8$ –17/group). (D and E) RV/LV + S and Lung/TL ratio analysis ($n = 17$ –29/group). (F and G) Cardiomyocyte CSA and RV fibrosis ($n = 7$ –9/group). (H and I) Mean Lumen/Total ratio and mean medial thickness in small vessels (<50 μ m) analysis ($n = 5$ –11/group). Results are presented as the mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. C; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. M. Two-way ANOVA repeated measures test was used for comparisons in panels (B and C), and two-way ANOVA was used for all the other parameters presented.

Figure 4 Acute and chronic treatment with hUcn-2 results in improved pulmonary arterial function in MCT-induced PH. (A and B) Relaxation in isolated pulmonary arterial rings from Control (C) and MCT-injected rats (M) pre-contracted with phenylephrine (10^{-5} M) in response to vehicle (Veh) and hUcn-2 (10^{-9} to 5×10^{-7} M; left panel). Black arrowhead on panel (A) represents incubation with 10^{-5} M of phenylephrine. (C) EC₅₀ for hUcn-2 in C and M groups ($n = 7$ /group). (D and E) Relaxation in isolated pulmonary arterial rings from C, CU, M, and MU rats pre-contracted with phenylephrine (10^{-5} M) in response to an acetylcholine dose-response (10^{-9} to 10^{-5} M; left panel). Black arrowhead on the panel (D) represents incubation with 10^{-5} M of phenylephrine. (F) EC₅₀ for acetylcholine in C, CU, M, and MU groups ($n = 9$ – 17 /group). Results are presented as the mean $\pm$ S.E.M. $**P < 0.01$, $***P < 0.001$ vs. line targeted groups in panels (B and E). $***P < 0.001$ vs. C; $###P < 0.01$ vs. M in panels (C and F). Two-way ANOVA repeated measures was used for comparisons in panels (B and E), while two-way ANOVA was used for panel (F). Student t-test was used for panel (C).

rings isolated from control animals, while there was no inhibitory effect in the MCT group with hUcn-2 pre-treatment (50 and 500 nM) (see [Supplementary material online, Figure S4](#)). We found compromised relaxation in a dose–response test to acetylcholine in pulmonary arteries isolated from MCT animals and treating animals with hUcn-2, while not changing phenylephrine-induced maximal tension (data not shown), significantly enhanced endothelial function, by increasing the maximal response to acetylcholine ([Figure 4D and E](#)). Furthermore, hUcn-2 decreased the EC₅₀ ([Figure 4F](#)), increasing receptor sensitivity to acetylcholine.

3.5 MCT-induced RV dysfunction is attenuated with hUcn-2 treatment

Pulmonary flow compromise with subsequent RV overload results in RV dysfunction. Likewise, echocardiographic analysis revealed a deteriorated pulmonary flow with the presence of mid-systolic notching ([Figure 5A](#), first panel) in the M group, where pulmonary artery acceleration time normalized to ejection time ratio (PAAT/PAET) and pulmonary artery velocity-time integral (PAVTI) were decreased ([Figure 5B and C](#)). TAPSE was also decreased ([Figure 5D](#)). hUcn-2 therapy improved pulmonary flow and increased TAPSE. RV catheterization showed that MCT-induced PH resulted in RV dysfunction 3 weeks after MCT administration ([Figure 5E](#)). hUcn-2 treatment reduced PVR ([Figure 5F](#)), and this resulted

in decreased RV end-systolic pressure (RVSP, [Figure 5G](#)), consistent with lessened RV hypertrophy, RV dilation (end-diastolic volume, EDV, [Figure 5L](#)) and RV dysfunction as shown by the increase of EF ([Figure 5H](#)) and CO ([Figure 5I](#)). Additionally, hUcn-2 therapy attenuated diastolic dysfunction, with reduced filling pressures, improved relaxation, and decreased diastolic stiffness, quantified by lower end-diastolic pressure (RVDP, [Figure 5J](#)), reduced isovolumic relaxation time constant (τ , [Figure 5K](#)), and decreased end-diastolic elastance (Eed, [Figure 5M](#)), respectively. Additionally, treated animals demonstrated improved systolic function, with decreased arterial elastance (Ea) while preserving contractility (Ees) resulting in improved RV-PA coupling (Ees/Ea) ([Figure 5N](#)). Intrinsic diastolic RV function, as measured in isolated skinned cardiomyocytes ([Figure 5O](#)), was also improved with hUcn-2 treatment, once the increase in passive tension at several sarcomeric lengths ([Figure 5P](#)) was attenuated in MU animals. Similar to the RV, hUcn-2 treatment improved global LV function, recovering both systolic and diastolic function (see [Supplementary material online, Figure S5](#)).

3.6 Molecular alterations in MCT-induced PH are reduced with hUcn-2 treatment

Rats with MCT-induced PH displayed increased brain natriuretic peptide (BNP) ([Figure 6A](#)) and endothelin-1 (ET-1) ([Figure 6B](#)) RV expression – peptides that are associated with cardiac overload and hypertrophy. Moreover, increased expression of apoptotic markers,

Figure 5 Treatment with hUcn-2 attenuates RV dysfunction in MCT-induced PH. (A) Representative figures of the echocardiographic analysis. (B–D) PAVTI, PAAT/PAET, and TAPSE analysis. (E) Representative pressure–volume loops of the different experimental groups. (F–N) PVR, RVSP, EF, CO, RVDP, Tau, end-diastolic volume, end-diastolic stiffness (Eed), and end-systolic elastance normalized to pulmonary artery elastance (Ees/Ea) ratio analysis in all experimental groups ($n = 10$ – 18 /group). (O and P) Representative single isolated skinned cardiomyocyte and passive tension (PT) in cardiomyocytes isolated from the RV of all groups ($n = 10$ – 18 /group, $n \approx 30$ cardiomyocytes/group). Results are presented as the mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. line targeted groups (panels B–D) and vs. C in panels (F–N and P); # $P < 0.05$; ### $P < 0.01$; #### $P < 0.001$ vs. M in panels (F–N) and vs. MU in panel (P). Two-way ANOVA repeated measures test was used for comparisons in panels (B–D and P), while two-way ANOVA was used for all the other parameters presented.

namely caspase-3 and caspase-8, was also observed in MCT animals (Figure 6C). Treatment with hUcn-2 reduced the expression of these markers of cardiac damage. The increase in RV fibrosis seen in MCT rats was paralleled by augmented levels of RV collagen type III alpha 1

(Col3A1) mRNA (Figure 6D), which were reversed by hUcn-2 treatment. In MCT-induced PH we also observed a shift towards β -myosin heavy chain (MHC) mRNA amplification and corresponding decline in α MHC, resulting in marked increase in the β/α MHC ratio in MCT

Figure 6 Chronic hUcn-2 treatment attenuates molecular alterations in experimental PAH-induced RVF. (A–F) BNP, ET-1, caspase-3 and -8, Col3A1, MHC, and HIF-1 α mRNA expression in the RV of Control and MCT-injected rats with (CU/MU) and without (C/M) hUcn-2 treatment ($n = 5$ –7/group). (G) Protein phosphorylation of p42/44 (Thr202/Tyr204) and p38 (Thr180/Tyr182) in the RV of all groups. (H and I) Analysis of pro-survival pathways in the RV of all experimental groups, with Bax/Bcl-2 ratio and phosphorylation of Akt (Ser473), GSK-3 β (Ser9), and STAT3 (Tyr705) ($n = 6$ –8/group). Results are presented as the mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. C; # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$ vs. M. Two-way ANOVA was used for all comparisons.

animals (Figure 6E). hUcn-2 therapy completely reversed the shift in MHC isoform expression. Furthermore, impaired RV function in MCT animals, underlined by decreased CO and RV perfusion, resulted in the up-regulation of hypoxia-inducible factor 1- α (HIF-1 α) mRNA (Figure 6F), and again, hUcn-2 administration abolished this increase. Western blot analysis showed reduced activation of downstream mitogen-activated protein kinase (MAPK) pathways

in the MCT group, with decreased phosphorylation of p-42/44 and p38 proteins. Chronic treatment with hUcn-2 restored the levels of these proteins (Figure 6G). The apoptotic bcl-2 like protein-4 normalized to B-cell lymphoma 2 (Bax/Bcl-2) ratio was increased in the MCT group, and treatment with hUcn-2 also abolished this rise (Figure 6H). Moreover, pro-survival signalling in the RV was diminished in MCT-induced PH, with decreased protein phosphorylation of

Figure 7 Chronic treatment with hUcn-2 decreases RV hypertrophy and fibrosis secondary to RV pressure overload. (A) Diagram depicting the PAB experimental protocol. (B) Representative echocardiographs (first line PSAX, parasternal short axis view; second line A4C, apical four chamber view), showing interventricular septum (white arrow heads) and RV diameter (white double arrow) in Sham and PAB rats with (BU) and without (S/B) hUcn-2 treatment. Representative H&E and picosirius red staining of RV samples (third and fourth lines) of all groups. Scale bars represent 50 µm (400× magnification) in H&E staining, and 25 µm (200× magnification) in picosirius red staining. (C) CO analysis in all groups. (D) Pulmonary artery gradient (PAG) across the PAB in both banding groups. (E and F) RVEDD and RV/LV + S ratio analysis. (G and H) RV cardiomyocyte CSA and RV fibrosis analysis in all groups ($n = 7-12$ /group). (I) Passive tension (PT) in cardiomyocytes isolated from the RV of all groups ($n = 7-12$ rats/group, $n \approx 30$ cardiomyocytes/group). Results are presented as the mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. S; # $P < 0.05$; ## $P < 0.01$ vs. B in panels (F–H) and vs. BU in panel (I). One-way ANOVA repeated measures test was used for comparisons in panels (I), while one-way ANOVA was used for all the other parameters presented.

glycogen synthase kinase 3 beta (GSK3 β) and signal transducer and activator of transcription 3 (STAT3), which was reversed by hUcn-2. No differences were observed in protein kinase B (AKT) phosphorylation (Figure 6I).

3.7 hUcn-2 treatment attenuated PAB-induced RV hypertrophy

Using the PAB model (Figure 7A), the therapeutic effects of hUcn2 on the RV, independent from its action on the pulmonary vasculature, were

investigated. PAB resulted in RV dilation, as judged by an increase in right ventricle end-diastolic dimension (RVEDD) (Figure 7E), hypertrophy, as evidenced by a higher RV/LV + S ratio (Figure 7F), and increased cardiomyocyte CSA (Figure 7G) and fibrosis (Figure 7H). hUcn-2 decreased RV hypertrophy and fibrosis relatively to untreated animals with the same degree of constriction (similar pulmonary artery gradient, Figure 7D), without compromising cardiac function (Figure 7C and see Supplementary material online, Figure S6). Finally, treatment with hUcn-2 attenuated the increase in passive stiffness observed in animals with PAB (Figure 7I).

4. Discussion

This study evaluated the expression of the Ucn-2/CRHR2 system in PAH and investigated the effect of hUcn-2 treatment in experimental PAH and RV hypertrophy. We found that Ucn-2 expression was increased in the RV of MCT-injected rats and patients with RVF, and that chronic hUcn-2 therapy attenuated experimental MCT-induced PH, as evidenced by beneficial effects on morbidity, exercise capacity and pulmonary and RV remodelling and dysfunction. Additionally, hUcn-2 treatment had direct effects on RV structure and function in a model of pressure loading of the RV without PAH.

Recent studies in experimental HF have shown favourable effects of Ucn-2 treatment in pacing-induced HF,^{13,14,32,33} myocardial infarction¹⁵ and systemic hypertension.²⁴ Beneficial effects in healthy volunteers and patients with acute¹⁷ and chronic HF^{16,34} have further excited interest in the therapeutic potential of the urocortins, although whether or not the increased Ucn-2 circulating levels is a cardioprotective response is yet to be understood.

Our results show elevated RV expression and plasma concentrations of Ucn-2 in MCT-induced PH, possibly in response to myocardial stress. Inversely, there was a decrease in CRHR2 mRNA levels in the RV, as observed in HF.³⁵ Interestingly, CRHR2 protein expression in MCT was increased in the RV. This mRNA/protein mismatch suggests a down-regulation of CRHR2 mRNA that precedes any decline in CRHR2 protein expression, possibly due to a decrease in receptor endocytosis.³⁶ In adipose tissue from animals subjected to hypoxia, both CRHR2 mRNA and protein levels are increased acutely, and while receptor protein expression remains elevated over 10 days post-hypoxia, CRHR2 mRNA expression gradually decreases to pre-hypoxia levels by day 10.³⁷ In human RVF, we found an increase of Ucn-2 mRNA and CRHR2 protein expression compared with the non-failing RV, similarly to our animal model. We were unable to find significant differences in the plasma levels of Ucn-2 in human PAH. Only three studies have measured plasmatic Ucn-2 levels in human patients with cardiovascular disorders, reporting significantly different (up to 1000-fold) concentrations in their cohorts.^{18–20} However, Ucn-2 seems to be activated on circulating and inflammatory cells (buffy coat), which mediate vascular and myocardial remodelling mechanisms.³⁸ Also, we did not find any differences in the lung expression of Ucn-2 and CRHR2 in experimental PAH, suggesting a more profound dysregulation of Ucn-2/CRHR2 signalling in the RV as compared with the lung.

Despite the gender differences observed in other peptides from the Ucn family and CRHR2,^{39–41} there were no gender-related differences in plasma Ucn-2 levels in patients with heart disease,^{19,21} nor in the role of CRHR2 in HF.²⁰ This was also true in our small cohort of patients.

We next analysed the effects of chronic hUcn-2 therapy in MCT-induced PH, and observed improvements in weight gain in the MU group, paralleled by an increase in exercise capacity, suggesting an integrated protective effect of hUcn-2 in the cardiovascular system and skeletal muscle. Exercise capacity, evaluated clinically by the 6-min walk test, and emulated here by exercise capacity testing, integrates the function of the whole cardiopulmonary system and has strong prognostic significance.¹ Also, disease progression is associated with increased mortality in PAH, and contains major prognostic relevance,⁴² therefore our findings that a lower percentage of MU rats achieved the defined end-points suggests a positive effect on future mortality.

Some of the beneficial Ucn-2 effects in HF result from direct actions on cardiomyocytes, however, they may be more pleiotropic.⁸ Indeed, hUcn-2 treatment resulted in the attenuation of both RV and pulmonary

vascular remodelling. RV dilation and hypertrophy were significantly reduced with hUcn-2 treatment, in association with decreased cardiomyocyte size and RV fibrosis. These results are consistent with recent observations in which Ucn-2 reduced post-infarct remodelling,¹⁵ and retarded the development of hypertension-induced LV hypertrophy.²⁴ Similarly, decreased pulmonary arterial small artery remodelling after hUcn-2 treatment led to a reduction in PVR and RV afterload, which explains, in part, the reduction in RV remodelling.

The observed RV dysfunction in MCT rats is consistent with decompensated RV hypertrophy, where compromised systolic flow is associated with elevated RVSP and correlates with decreased survival in PAH.⁴³ In MCT-induced PH, hUcn-2 improved pulmonary arterial flow and decreased RV afterload. TAPSE, an indicator of RV contractile function, was also improved. MCT-injected animals exhibited severe cardiac dysfunction, with compromised CO, EF and increased RVSP, which were both ameliorated by hUcn-2. We also observed that MCT-induced PH was associated with RV-PA uncoupling, due to an insufficient increase in contractility to match the increase in afterload.⁴⁴ Abnormal RV-PA coupling is a major determinant of RV HF and one of the leading causes of death in PH,⁴⁵ and its evaluation may help identify at-risk-patients or guide the timing of therapeutic interventions in PAH.⁴⁶ Remarkably, hUcn-2 decreased pulmonary elastance (Ea) without compromising RV contractility (Ees), resulting in an improved RV-PA coupling. Diastolic dysfunction was also attenuated by hUcn-2, as shown by decreased stiffness and RV diastolic elastance. At the cellular level, hUcn-2 reduced passive tension measured in isolated RV cardiomyocytes, contributing to the decreased stiffness and improved diastolic function observed.

Additionally, we observed an acute effect of hUcn-2 in modulating pulmonary artery vasomotor status, which probably contributed to a change in PVR. Several endothelium-independent and -dependent mechanisms have been proposed to describe Ucn-2-mediated vasodilation.^{22,23,47} Our analysis of acute hUcn-2 effects in pulmonary arterial rings from MCT-injected animals demonstrated a significant endothelium-independent vasodilatory effect, although attenuated when compared with control vessels. This can be an advantage in PAH which is associated with impaired endothelial homeostatic mechanisms. Furthermore, Ucn-2 induces a potent prolonged arterial vasodilation without causing significant tachyphylaxis,²³ which is important especially when considering its potential application as a chronic PAH therapy.

Myocardial remodelling, increased wall stress, hypoxic damage and apoptosis in MCT-induced PH are correlated with increased RV mRNA expression of damage markers,^{28,48} which was reduced by hUcn-2 therapy, either by directly acting on these signalling pathways, or indirectly by attenuating RV dysfunction. Moreover, the decreased phosphorylation of both ERK 1/2 and p38 MAPK likely relates to the advanced stage of HF⁴⁹ in our model, and its activation by hUcn-2,⁵⁰ may be a preventive mechanism. Akt was not affected by any drug administered (MCT/hUcn-2), as previously reported in.⁵¹ Increased STAT3 and GSK3 β phosphorylation and reduced Bax/Bcl-2 ratio in MU rats match what has been previously described *in vitro*,^{52,53} demonstrating the cardioprotective Ucn-2 properties.

Recently, it was demonstrated that plasma Ucn-2 levels significantly increase in human and experimental LVHF. Additionally, continuous CRHR2 agonist infusion (100 μ g/kg/day during 4 weeks) in control mice increased plasma Ucn-2 in a similar degree to that observed in mice with pressure overload-induced HF, resulting in cardiac dysfunction without having a significant effect on systolic blood pressure.²⁰ These findings suggest that higher doses of chronic Ucn-2 administrations without vaso-relaxation may have cardiotoxic effects, activating chronically the PKA,

CaMKII, and AKT signalling pathways. In our experimental study, although Ucn-2 therapy modulated Ucn-2/CRHR2 expression in the RV, there was no significant increases in plasmatic Ucn-2, which could impair cardiac function.

The Ucn-2 effects can be explained by pulmonary vascular mechanisms; however, it was unclear whether hUcn-2 has any myocardial-specific effects. Indeed, there is some concern over data suggesting that the Ucn-2 may induce hypertrophic responses in cardiac myocytes.²⁰ Thus, similarly to a previous work by our group,²⁸ we decided here to use a mild PAB model, as it better replicated the degree of overload observed in MCT-induced PH, without pulmonary vascular involvement.²⁹ Other works showed that adapted RVs with increased afterload but with compensated function have significant RV structural changes^{54–57}; however, specific comparisons with this model can be difficult to make, as significant variances can be obtained in different conditions. Interestingly, hUcn-2 attenuated RV hypertrophy and fibrosis, without compromising function, indicating that hUcn-2 has a direct effect on the RV contributing to the improved RV function observed in MU animals, at least in hUcn-2 dose used in MCT protocol. As in the MCT model, constriction of the pulmonary artery promotes not only structural changes, but also intrinsic alterations.^{29,58} Interestingly, hUcn-2 decreased passive tension development, contributing directly to attenuation of diastolic dysfunction in these animals, which could be the tipping point for RVF in PAH.⁵⁸

One limitation of our work is the lack of categorization of PAH patients accordingly to their RV phenotype with an individualized clinical follow-up. Indeed, the low levels of plasma Ucn-2 may relate to a CRV state in these patients. However, there is an association between Ucn-2/CRHR2 expression and RV dysfunction. In the experimental setting, the limitations include the lack of investigation of subcellular mechanisms underlying the actions of Ucn-2 in the pulmonary vasculature, and this should be the subject of further research. Although not all characteristics of PAH are simulated by the MCT model, it shares many characteristics with primary and secondary human PH, including pulmonary vascular remodelling, and RV and endothelial dysfunction³⁸ and hUcn-2 ameliorated most of these parameters. Additionally, at 14 days after MCT administration animals exhibit RV hypertrophy, vascular remodelling, and pulmonary flow compromise, suggesting that hUcn-2 reverses already established PAH, which is clinically important.¹ Also, LV dysfunction which is present in advanced stages of PAH⁵⁹ was attenuated by hUcn-2, underscoring the putative translational implication. In PAB protocol, the underlying mechanisms of the myocardial-specific effects in RV should as well be a subject of further study.

In conclusion, this study demonstrated that Ucn-2/CRHR2 signalling has an important role in PAH and RV dysfunction, given that levels of Ucn-2 and its receptor are altered in human and experimental PAH. Additionally, hUcn-2 treatment improves cardiopulmonary structure and function, once it attenuates PAH and RV dysfunction in MCT-induced PH, has direct anti-remodelling effects on the pressure-overloaded RV, and improves pulmonary vascular function.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

The authors would like to acknowledge the support from the Histology and Electron Microscopy Service (HEMS) core facility at i3S – Instituto

de Investigação e Inovação em Saúde, University of Porto for the help with histological immunohistochemistry for CRHR2 receptor protein.

Conflict of interest: none declared.

Funding

This work was supported by Portuguese Foundation for Science and Technology (FCT) through Grant UID/IC/00051/2013 (COMPETE_2020, POCI) and projects FCOMP-01-0124-FEDER-028695, FEDER, COMPETE, Ref: FCT-PTDC/DTP-FTO/0130/2012; NETDIAMOND (POCI-01-0145-FEDER-016385) and DOCnet (NORTE-01-0145-FEDER-000003, NORTE_2020, under PORTUGAL_2020 Partnership). PMF and RA are supported by FCT (SFRH/BD/87714/2012 and SFRH/BD/96403/2013, respectively).

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Online supplements to:

Urocortin-2 improves right ventricular function and attenuates pulmonary arterial hypertension

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Supplementary methods

All patients or their legal representatives (in the case of autopsy) gave informed consent before the beginning of the study in accordance to the declaration of Helsinki, and institutional guidelines were approved by Institut Universitaire de Cardiologie et de Pneumologie de Québec (CER-20773). The usage of human tissues follow recommendations published,¹ in collaboration with the Institut Universitaire de Cardiologie et de Pneumologie de Québec site of the Respiratory Health Network tissue bank of the Fonds de recherche du Québec – Santé (FRQS)(www.tissuebank.ca).

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 (PTDC/DTP-FTO/0130/2012; 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*.

Human samples

Human RV samples were collected from patients with and without PAH at the time of cardiac surgery (see Table S1), heart transplantation or autopsy (performed within 3 h of death). Samples were categorized according to clinical history and the tricuspid annular plane systolic excursion (TAPSE), and designated as normal RV (NRV) or RV failure (RVF) as previously reported.² Human lung samples were obtained from PAH patients at the time of lung transplantation and from non-PAH controls during tumour resection from non-cancerous segments.³ Blood samples were also collected from PAH patients and age-matched controls as previously described.⁴

Animal models and experimental design

PAH was induced as previously described.⁵ Seven-week-old male Wistar rats (Charles River Laboratories, 180-200 g) randomly received an injection of monocrotaline (MCT, 60 mg/kg, s.c., Sigma-Aldrich) or an equal amount of vehicle. Two weeks after MCT/vehicle administration, animals were further randomly received hUcn-2 (Bachem) at a dose of 5 ug/kg (bi-daily, i.p.) or an equal amount of vehicle (0.9% NaCl) for 10 days, resulting in four groups: Ctrl+vehicle (C); Ctrl+hUcn-2 (CU); MCT+vehicle (M); MCT+hUcn-2 (MU). After the treatment period, animals were submitted to exercise testing and echocardiographic and invasive haemodynamic analysis, with subsequent sample collection for *in vitro* functional studies, morphological, histological, and molecular analysis, as detailed in supplemental material. To determine whether MCT-induced PH was already present prior to therapy, an additional group underwent the same experimental protocol and was evaluated at an earlier time point (14 days) (see Figure S7 and S8).

Another group of animals was subjected to pulmonary artery banding (PAB), as previously described,⁶ applying a pulmonary artery constriction resulted in a degree of hypertrophy and RV overload identical to the MCT-induced PH model (see Figure S6), and submitted to the same randomization, time points, and chronic treatment protocol. Seven-week old male Wistar rats (Charles River Laboratories, 180-200 g) were sedated, anaesthetized and monitored as above mentioned (for the haemodynamic evaluation). The animal was placed in right lateral decubitus, the left thorax was shaved, depilated and wiped clean with iodine solution. Under a surgical microscope (Wilde M651, Leica microsystems), a small incision was created at the axillary level, the pectoral muscles were carefully dissected and retracted, and the thorax was opened at the second intercostal space. The thymus was moved and the pulmonary artery visualized. Using blunt forceps, the pulmonary artery was dissected free

from both sides and a perforated spinal cord hook (Fine Science Tools) was used to pass a 6-0 prolene suture around the pulmonary artery. A 16-gauge needle with a blunt tip was placed parallel to the pulmonary artery, and the suture was quickly tied leaving a 1.65 mm constriction. The thorax was closed with 5-0 prolene, taking care not to cause pneumothorax, the pectoral muscles were put back in place and the skin was closed using 7-0 prolene. The suture site was infiltrated with lidocaine (1 %) and a subcutaneous injection of saline was administered to compensate for fluid losses, as well as analgesia (morphine, 4 mg/kg every 4 hours for the first 48 hours). Anaesthesia was interrupted and the animals were allowed to recover until full motion was achieved, and placed in a clean cage on top of a heating pad with easy access to food and water. Suture integrity was checked for the next 3-4 days to ensure proper wound closure. Sham animals underwent the exact same protocol, except that the suture around the PA was loosely tied, and were not failed PAB attempts. No mortality was observed 3 weeks after PAB. Two weeks after PAB, animals randomly received hUcn-2 or vehicle for 10 days, resulting in three groups: Sham + vehicle (S); PAB + vehicle (B) and PAB + hUcn-2 (BU). Sampling was performed as in the MCT protocol.

Drugs preparation

A solution of MCT was prepared wherein 300mg was dissolved in 2.36mL of 1M HCl, diluted with distilled water to ~1.60mL, neutralized with 1.52mL of 1M NaOH, and pH adjusted to 7.00.

The hUcn-2 dosages, administration routes and treatment duration were selected based on previous studies⁷ and preliminary dose-finding experiments (unpublished data). A stock solution of hUcn-2 was initially prepared by dissolving 1mg in 1mL of NaCl, which was further diluted with NaCl to achieve a final working concentration of 0.002 mg/mL.

Survival analysis

The defined end-point for the survival analysis was a 20% loss of body weight plus oedema/ascites and decreased movement. When this end-point was reached, animals were euthanized with an anaesthetic overdose (sodium pentobarbital 100mg/kg, Eutasil, CEVA). Only those animals reaching the end-point before evaluation at study-end were considered valid and used in the survival analysis.

Exercise capacity test

Animal exercise capacity was tested on a close-chamber treadmill set with electric stimulation (LE8710R, Columbus Instruments) coupled to a gas analyser. Peak effort and endurance tests were evaluated in different sets of animals. To assess maximal oxygen consumption (VO_2max), treadmill velocity was increased after an initial adaption period at 9m/min to 18m/min and then stepped up by 3m/min every minute, at a treadmill inclination of 15°,⁸ whereas in endurance testing, initial speed was set at 10m/min, with increases of 5m/min every 5 minutes, for 30 minutes or until the animal fatigued.⁹ Both tests were performed 13 (prior to treatment group allocation; baseline) and 23 days after PAH induction.

Echocardiography

Echocardiographic analysis was performed 13 days (baseline) and 24 days after PAH induction as previously described.⁵ Briefly, animals were lightly anaesthetized (inhalation of 8% sevoflurane in a closed chamber for induction, and 2–3.5% for maintenance through a nose cone) and placed in a left lateral decubitus position on a heating pad with homeothermic control through a rectal probe. The thorax was shaved, cleaned and scanned with a 15 MHz linear probe (15L8 probe coupled to an Acuson Sequoia C512 echocardiography system, Siemens) for determination of pulmonary flow and RV structure. Acquisitions were averaged over three consecutive heartbeats by a researcher blinded to the experimental group. Pulmonary artery acceleration time (PAAT), pulmonary artery

ejection time (PAET) and the pulmonary artery velocity-time integral (PAVTI) were evaluated through conventional Doppler in parasternal short-axis view, and the PAAT/PAET ratio calculated. RV end-diastolic dimension (RVEDD), right atrial area (RAA), and TAPSE were obtained in an apical 4-chamber projection.

Invasive haemodynamic evaluation

Following echocardiographic evaluation, the animals were intubated using a 14-gauge catheter and ventilated (MouseVent G500, Kent Scientific) with 100% O₂, with tidal volume and respiratory rate adjusted to animal weight, and monitored for peripheral oximetry, heart rate (HR), capnography, and respiratory rate (PhysioSuite, Kent Scientific). Anaesthesia was titrated according to toe-pinch reflex. The animals were placed on their right side, the internal femoral vein catheterized for fluid administration, and a left thoracotomy performed. High fidelity pressure-volume catheters were inserted through the apex of the RV and LV and positioned along the long axis (SPR-869 and SPR-847, for the RV and LV, respectively, Millar Instruments, Texas, USA), and a flow probe (MA2.5PSB, Transonic Systems) implanted around the ascending aorta for CO acquisition and field inhomogeneity normalization. Baseline and inferior vena cava occlusion recordings were obtained, and a bolus injection of 50 μ L 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 anaesthetic overdose (sodium pentobarbital 100mg/kg, Eutasil, CEVA), blood samples were taken into EDTA tubes, the animals exsanguinated and heart and lungs collected *en bloc* for subsequent histological and molecular analysis.⁵

Morphometric and histological analysis

Following removal, the lungs, RV and LV + septum (LV + S) were carefully separated and weighed before being flushed with cold physiological salt solution (PSS, 130mM NaCl, 4.7mM KCl, 1.18mM KH₂PO₄, 1.17mM MgSO₄, 14.9mM NaHCO₃, 5.5mM glucose, 0.026mM EDTA and 1.6mM CaCl₂) to remove excess blood. RV and lung samples were collected, snap frozen in liquid nitrogen, and stored at -80°C. For mRNA quantification, samples were submerged in RNA stabilization reagent (RNAlater, Qiagen). Tibia length was measured for weight normalization.

For histological analysis, samples of RV and lung tissue were submerged in fixative solution (10% formaldehyde) and dehydrated before undergoing diaphanization and impregnation in liquid paraffin. Four μ m sections of RV were cut and stained with haematoxylin and eosin (HE) to evaluate cardiomyocyte morphology, and with Picro Sirius Red to quantify fibrosis. Sections were digitally photographed (Olympus XC30, Olympus) and analysed using imaging software (Cell[^]B, Olympus).⁵

For the lung tissue, HE-stained tissue sections were scanned at 40X magnification using the Philips IntelliSite UltraFast Scanner system and supported by QuPath (Version 0.1.2) software analysis package.¹⁰ Morphometric analysis was carried out on each of the lung sections, where all vessels/arterioles present in 10 fields/section (10 mm²) were categorized into small (15-50 μ m), medium (50-100 μ m) and large (> 100 μ m) and measurements for the total and lumen vessel diameter recorded, essentially as previously described.¹¹ Based on these measurements, values for the Lumen/Total Vessel Diameter Ratio, Medial Thickness (μ m) and Degree of Vessel Occlusion (%) were calculated. Additionally, Vessel Occlusion was categorized as: no evidence of neointimal formation (open); partial (<50%) luminal occlusion; and full-luminal occlusion (closed).¹²

Assessment of isolated pulmonary artery endothelial function and vascular acute analysis

After heparinization, euthanasia was achieved with anaesthetic overdose (sodium pentobarbital 100mg/kg, Eutasil, CEVA), the heart and lung removed *en bloc*, and submerged in cold physiological salt solution (PSS, 130 mM NaCl, 4.7 KCl, 1.18 mM KH₂PO₄, 1.17 mM MgSO₄, 14.9 mM NaHCO₃, 5.5 mM glucose, 0.026 mM EDTA and 1.6 mM CaCl₂). The left upper lung was used to isolate second-generation pulmonary arteries (200 – 400µm diameter). Arterial rings (2 ± 0.2 mm) were cut and mounted between two metallic pins in a horizontal bath myograph system (DMT Myograph 720 MO, ADInstruments) at 95% O₂ / 5% CO₂, a constant temperature of 37°C and pH of 7.4. Following a stabilization period, normalization of each arterial ring to its optimal length (length-tension curve to transmural target pressure) and maximum tension development was assessed with 80 mM KCl (KPSS) solution.⁵

For the determination of the acute vasodilatory effects of hUcn-2, arterial rings were contracted with phenylephrine (10⁻⁵ M) and allowed to stabilize, after which a dose response to hUcn-2 (10⁻⁹ to 5x10⁻⁷ M) or vehicle (ddH₂O, equal volume) was performed. The dose response to hUcn-2 was also carried out in endothelium-denuded vessels, affected by passing a 7-0 silk suture over the intimal surface of the lumen to remove the endothelial layer. Maximal relaxation to hUcn-2 (E_{max}) and the concentration of hUcn-2 required for 50% of the maximal response (EC₅₀) were calculated.

For the determination of the acute vasoconstriction-inhibiting effects of Ucn-2, the arterial rings were firstly contracted with phenylephrine (10⁻⁵ M), followed by relaxation via 15-minutes incubation with 50nM hUcn-2 (5 x 10⁻⁸ M), - the closest concentration to the EC₅₀ (63 nM, or 10^{-7.2} M) of control arterial rings - and then contracted again with phenylephrine (10⁻⁵ M). Contraction inhibition was calculated as the ratio of maximum developed tension between the second and the first phenylephrine contraction. An alternative set of arterial rings isolated from MCT animals was pre-incubated with the higher dose of hUcn-2 (500 nM, 5 x 10⁻⁷ M).

Pulmonary artery endothelial function was assessed by attaining a dose-response curve to acetylcholine (10⁻⁹ to 10⁻⁵ M) after pre-contraction with phenylephrine (10⁻⁵ M). Maximal relaxation to acetylcholine and EC₅₀ were calculated. Analysis was performed offline (LabChart 7 Pro, ADInstruments).

In vitro studies in isolated skinned cardiomyocytes

RV samples (stored at -80°C) 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 403 A, Aurora Scientific) and a length controller (model 315 C-I, Aurora Scientific). Cell length was digitally adjusted through custom-designed software (series 600A digital controller, Aurora Scientific), and steady-state passive force measured at increasing sarcomere lengths (1.8 - 2.3 µm). Force measurements were normalized to each cardiomyocyte cross-sectional area (CSA) and a minimum of three cells per animal measured.⁶

Quantitative RT-PCR, immunoblotting, immunochemistry and enzyme-linked immunosorbent assay

For human samples, total RNA was isolated from tissue using the RNeasy micro kit according to the manufacturer's instruction, and mRNA reverse transcribed and amplified using one-step kit TaqMan probes. qRT-PCR was performed with the ABI Prism 7900 Sequence Detector (Applied Biosystems), and 18 s used as a housekeeping gene. The relative expression level of Ucn-2 and CRHR2 was determined by the 2^{-ΔCt} *100.

For rat samples, total RNA was extracted using the RNeasy Mini Kit according to manufacturer's instructions, and relative mRNA expression of Ucn-2, CRHR2, B-type brain natriuretic peptide (BNP), Endothelin-1 (ET-1), Hypoxia-inducible factor 1-alpha (HIF-1α), Collagen type III alpha 1 (Col3A1), Caspase-3 and -8, myosin heavy chain β (βMHC) and myosin heavy chain α (αMHC) 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 and normalized for the control group. All assays were performed in duplicate. Primers used were designed in-house (see Table S2).

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 4°C, 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 4°C with primary antibodies (see Table S3). 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). Human RV CRHR2 expression was quantified using a horse-radish peroxidase conjugate secondary antibody (Bio-Rad) and ChemiDoc system, and normalized for total protein content (Amido Black, Sigma Aldrich).

For immunohistochemistry, RV sections were deparaffinized and rehydrated. Antigen Retrieval was performed according to the manufacturer in a steamer. Sections were incubated overnight at 4° with the primary antibody anti- CRHR2 (Abcam, ab203585) at 1:250, diluted in antibody diluent (Thermofisher scientific, USA). Slides were then washed in TBS-T20 and incubated with POLYVIEW® PLUS HRP-DAB (Anti-Rabbit) Kit and counterstained with haematoxylin, provided in the kit. Positive and negative controls were included.

Blood collected from rats and humans was centrifuged and dispensed plasma assayed for Ucn-2 concentrations using solid-phase sandwich enzyme-linked immunosorbent assay according to manufacturer's instructions (SEC585Ra and SEC585Hu, Enzyme-linked immunosorbent assay kits for Ucn-2, Cloud-Clone Corp.). In addition, buffy coat was analysed for Ucn-2 expression in human samples.

Statistical analysis

The results are presented as the mean ± SEM. Survival analysis was performed in R (version 3.4.1) using PwrGSD package (version 2.0), whereas GraphPad Software v7 (GraphPad Software Inc., San Diego, CA, USA) was used in all other parameters. Two-way analysis of variance (ANOVA), with repeated-measures when suitable, was used to analyze most parameters with Tukey's method for post hoc comparisons between groups. Where appropriate, Student t-test or one-way ANOVA with Tukey's method for post hoc comparisons were used. Survival analysis was estimated according to the Kaplan-Meier method and comparisons were performed using a generalized Fleming-Harrington test ($p = 1$, $q = 1$) for nonproportional hazards, which gives more weight to later events. A P -value of <0.05 was considered to be statistically significant.

Table S1 Patient characteristics

	CRV (n=8)	RVF (n=8)	P-value
Female sex, %	75	60	N/A
Age, y	35 ± 7	56 ± 9	NS
<u>Subclass of PAH</u>			
IPAH	-	1 (12.5)	-
SSc-PAH	-	3 (37.5)	-
Other	-	4 (50)	-
<u>NYHA, %</u>			
I	50	-	-
II	50	-	-
III	-	25	-
IV	-	25	-
<u>3D Echo</u>			
LVEF, %	58 ± 2	56 ± 3	NS
TAPSE, mm	22 ± 2	13 ± 2	P<0.01
RVFS, %	54.5 ± 2.9	27.8 ± 4.1	P<0.01
<u>RHC</u>			
mPAP, mmHg	19.5 ± 0.5	44.0 ± 3.0	P<0.01
RAP, mmHg	7.5 ± 1.5	10.8 ± 3.9	NS
PCWP, mmHg	8.0 ± 3.0	10.3 ± 1.7	NS
CI, L/min/m ²	2.35 ± 0.17	2.41 ± 0.22	NS
PVR, dyne.s/cm	236 ± 63	646 ± 72	P<0.01

CI, cardiac index; CRV, compensated right ventricle; IPAH, idiopathic pulmonary arterial hypertension; SSc-PAH, systemic sclerosis associated pulmonary arterial hypertension; LVEF, left ventricle ejection fraction; mPAP, mean pulmonary artery pressure; NYHA, new York Heart Association classification functional classification; PCWP, pre-capillary wedge pressure; PVR, pulmonary vascular resistance; RAP, right atria pressure; RHC, right heart catheterization; RVF, right ventricle failure; RVFS, right ventricle fractional shortening; TAPSE, tricuspid annular plane systolic excursion. Values are presented as mean±SEM.

Table S2 Primer Sequence

Gene	Primer Sequence
GAPDH	P1 - 5'TGGCCTTCCGTGTTCCCTACCC ^{3'} P2 - 5'CCGCCTGCTTCACCACCTTCT ^{3'}
UCN-2	P1- 5'CGTTGGCATAACGCCTCAC ^{3'} P2- 5'GGACACAGAGCTGGGAGTTG ^{3'}
CRHR2	P1 - 5'TGCAACTCATCGACCACGAA ^{3'} P2- 5'CAGGTAGCAGCCTTCCACAA ^{3'}
ET-1	P1- 5'CGGGGCTCTGTAGTCAATGTG ^{3'} P2 - 5'CCATGCAGAAAGGCGTAAAAG ^{3'}
BNP	P1- 5'CAG AGC TGG GGA AAG AAG AG ^{3'} P2- 5'GGACCAAGGCCCTACAAAAGA ^{3'}
HIF-1 α	P1 - 5'TCATAGGCGGTTTCTTGTAGC ^{3'} P2 - 5'CTAACAAGCCGGAGGAC ^{3'}
Caspase-3	P1 - 5'CGGGTGCGGTAGAGTAAG ^{3'} P2 - 5'CTGGACTGCGGTATTGAGACA ^{3'}
Caspase-8	P1 - 5'ACCAAATGAAGAGCAAACCTCG ^{3'} P2 - 5'TTTTCTGTCCCGCATGTTGC ^{3'}
α MHC	P1 - 5'CAAGAAGAACTTGGTGCGCGGC ^{3'} P2 - 5'ATCGTGCATTTTCTGCTTGGC ^{3'}
β MHC	P1 - 5'AAGTCCTCCCTCAAGCTCCTAAGT ^{3'} P2 - 5'TTGCTTTGCCTTTGCCC ^{3'}
Col3A1	P1- 5'AGCATCCATCTTGCAGCCTT ^{3'} P2- 5'GCTCGGAATTGCAGAGACCT ^{3'}

BNP, brain natriuretic peptide; Col3A1, Collagen type III alpha 1; CRHR2, corticotrophin-releasing hormone receptor type 2; ET-1, endothelin 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HIF-1 α , hypoxia inducible factor 1 alpha; UCN-2, urocortin-2; α MHC, alpha myosin heavy chain; β MHC, beta myosin heavy chain.

Table S3 Primary antibodies

Protein	Antibody's Identification
Akt	Akt (pan) (C67E7), Rabbit mAb, Cell Signaling Technology
p-Akt	Phospho-Akt (Ser473) (587F11), Mouse mAb, Cell Signaling Technology
ERK1/2	p44/42 MAPK (Erk1/2) (3A7), Mouse mAb, Cell Signaling Technology
p-ERK1/2	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), Rabbit pAb, Cell Signaling Technology
p38	p38 α MAPK (L53F8), Mouse mAb, Cell Signaling Technology
p-p38	Phospho-p38 MAPK (Thr180/Tyr182) (D3F9), Rabbit mAb, Cell Signaling Technology
STAT3	Stat3 (124H6), Mouse mAb, Cell Signaling Technology
p-STAT3	Phospho-Stat3 (Tyr705) (D3A7), Rabbit mAb, Cell Signaling Technology
GSK-3 β	GSK-3 β (3D10), Mouse mAb, Cell Signaling Technology
p-GSK-3 β	Phospho- GSK-3 β (Ser9) (D3A4), Rabbit mAb, Cell Signaling Technology
Bax	Bax (D2E11), Rabbit pAb, Cell Signaling Technology
Bcl-2	Bcl-2 (124), Mouse mAb, Cell Signaling Technology
CRHR2	Anti-Corticotropin Releasing Factor Receptor 2, Rabbit pAb, Abcam
GAPDH	GAPDH (0411), Mouse mAb, Santa Cruz Biotechnology, Inc.
CAMKII	CaMKII (sc-5306), Mouse mAb, Santa Cruz Biotechnology, Inc.
p-CAMKII	Phospho-CaMKII (Thr286) (sc-32289), Mouse mAb, Santa Cruz Biotechnology, Inc.
PKA	PKA α (sc-28315), Mouse mAb, Santa Cruz Biotechnology, Inc.
p-PKA	p-PKA α (C-5) (sc-377575), Mouse mAb, Santa Cruz Biotechnology, Inc.

Akt, protein kinase B; Bax, Bcl-2 Associated X Protein; Bcl-2, b-cell lymphoma 2; CAMKII, calmodulin-dependent protein kinase II; CRHR2, corticotropin-releasing hormone receptor type 2; ERK1/2, extracellular-signal regulated kinases 1 and 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSK-3 β , glycogen synthase kinase 3 beta; p38, p38 mitogen-activated protein kinase; PKA, protein kinase A ; PLB, phospholamban; Serca2A, sarcoplasmic reticulum Ca²⁺-ATPase; STAT3, signal transducer and activator of transcript

Figure S1. (A, B) Correlation between mRNA expression of urocortin-2 (Ucn-2) in the RV of PAH patients and mean pulmonary arterial pressure (mPAP) and tricuspid annular plane systolic excursion (TAPSE). (C) Buffy coat from patients with PAH shows increased expression of UCN-2 compared to control samples (n=6-7/group). (D) Ucn-2 expression in the lung of PAH patients is not significantly decreased when compared to non-PAH lung samples (n=5-8/group). (E, F) Correlation between mRNA expression of Ucn-2 in the RV of monocrotaline (MCT) rats and right ventricular end-systolic pressures (RVESP) and ejection fraction (EF). (G) Correlation between mRNA expression of Ucn-2 in the RV of all rats and Ucn-2 plasmatic levels. (H) Plasmatic levels of Ucn-2 in MCT rats after 14 and 24 days of the MCT administration. (I) Correlation between Ucn-2 plasmatic levels and TAPSE in MCT animals. (J, K) Plasmatic levels and mRNA expression of Ucn-2 in male and female patients. Results are presented as the mean $\pm$ SEM. * $p<0.05$ vs CTRL in panel C, and vs C in panel H. Pearson correlation coefficient was used in panels A, B, E, F, G and I. Student t-test were performed to compare groups in panels C and D. One-way ANOVA was performed in panel H. Two-way ANOVA was used in panel J and K.

Figure S2. Immunohistochemistry for CRHR2 receptor protein in the right ventricular tissue of control and MCT-induced PH animals, confirming the presence of the receptor in heart tissue. Scale bars represent 200µm (100X magnification) in the left panels, and 50µm (400X magnification) in the right panels. Black arrowheads indicate zones of high intensity positive staining.

Figure S3. Morphometric analysis to evaluate vascular remodelling in all, small (15-50 μm) and medium (50-100 μm) pulmonary arterioles. **(A, E, I)** The degree of vessel occlusion of pulmonary arterioles was determined and vessels were categorised into Open (O; < 50%), Partially Closed (PC; ≥ 50%, <95%) or Closed (C; ≥ 95 %) vessels. In the all and small arterioles, the number of Open (O) and Partially Closed (PC) vessels in MCT-treated animals is significantly decreased relative to the CTRL group, whereas the number of Open (O) and Partially Closed (PC) vessels in MU animals is significantly increased relative to MCT animals. No differences were obtained in medium vessels. **(B, F, J)** In all and small arterioles, the Lumen/Total Ratio in MCT animals is decreased compared to CTRL rats, whereas in MU animals the Lumen/Total Ratio is significantly increased from MCT animals. No differences were obtained in medium vessels. **(C, G, K)** In all and small arterioles, the mean medial thickness in MCT animals is increased compared to CTRL rats, whereas, in small arterioles, the medial thickness from MU animals is significantly decreased compared to MCT animals. No differences were obtained in medium vessels. **(D, H, L)** In all and small arterioles, the mean degree of vessel

occlusion in MCT animals is decreased compared to CTRL rats, whereas in MU animals is significantly increased compared to MCT animals. No differences were obtained in medium vessels (n=5-11/group). Results are presented as the mean $\pm$ SEM. *p<0.05, **p<0.01, ***p<0.001 versus line targeted groups (panels A and I) and versus C in panels B-D and J-L; #p<0.05; ##p<0.01; ###p<0.001 versus line targeted groups (panels A and I) and versus M in panels B-D and J-L. Two-way ANOVA was used for all comparisons.

Figure S4. Vasodilation response to hUcn-2 is independent from the endothelium and pre-treatment with hUcn-2 inhibits phenylephrine-induced vasoconstriction in control vessels. **(A, B, D, E)** In control (n=6-8/group) and MCT animals (n=6-11/group), vasodilation response to hUcn-2 is independent from the endothelium, as both preserved and removed endothelium (Endo(+) and Endo(-), respectively) vessels pre-contracted with phenylephrine (10⁻⁵M) respond to human urocortin-2 (hUcn-2; 10⁻⁹ to 5x10⁻⁷M, left panel). **(C)** In control animals, EC₅₀ for hUcn-2 is not different in pulmonary arterial rings with (+) and without (-) endothelium (n=6-8/group). **(F)** In MCT rats, EC₅₀ for hUcn-2 in pulmonary arterial rings without endothelium (-) is lower, compared to vessels with endothelium (+) (n=6-11/group). **(G, H)** Pre-treatment with hUcn-2 (50 nM) inhibits phenylephrine-induced vasoconstriction in pulmonary arterial rings isolated from control animals. In MCT-induced PH pulmonary arterial rings, neither 50 nM, nor 500 nM exert an inhibitory effect on phenylephrine-induced vasoconstriction. Black arrowheads on the right panel represent incubation with 10⁻⁵M of phenylephrine, while grey arrowhead represents hUcn-2 treatment (n=6-8/group). Results are presented as the mean±SEM. **p<0.01, versus group C. Two-way ANOVA repeated measures was used for comparisons in panel A and D. Student t-test was used for panels C and F.

Student t-test (CTRL group) and One-way ANOVA (MCT group) was used for panel G.

Figure S5. hUcn-2 therapy improves left ventricular (LV) function in MCT-induced PH. **(A)** Representative pressure– volume loops of the different experimental groups. **(B)** LV end-systolic pressures were decreased in MCT animals, and treatment with hUcn-2 attenuated this alteration. **(C)** End-diastolic volume was decreased in MCT-induced PH and normalized with hUcn-2 treatment. **(D)** Isovolumic relaxation time constant (tau) was increased in MCT animals and normalized with hUcn-2 therapy. **(E)** Eed was increased in MCT-induced PH and normalized with treatment. (n=10-18/group). Results are presented as the mean $\pm$ SEM. *p<0.05,**p<0.01,***p<0.001 versus C; #p<0.05 versus M. Two-way ANOVA was used for all the parameters presented.

Figure S6. Pulmonary artery banding (PAB) results in similar degrees of RV hypertrophy and overload to the monocrotaline (MCT)-induced PH model. **(A)** Illustration of the placement of the catheters for the measurement of the pressure gradient (PAG) across the constriction. One of the catheters (PVR-1045) was introduced in the pulmonary artery and the other one in the RV. **(B)** PAB animals showed preserved ejection fraction, with an increase after hUcn-2 treatment. **(C, D)** PAB resulted in an increased end-systolic pressure (RVSP) in both PAB groups, without changing end-diastolic pressures (RVEP). **(E)** PAB animals present similar values of RVSP, pointing to similar degrees of RV pressure overload in both animal models. **(F, G)** PAB animals present values of RV/LV+S and RV/TL ratios identical to animals with MCT-induced PH, pointing to the same degree of hypertrophy in both animal models. **(H, I)** Histological analysis of cardiomyocyte cross sectional area (CSA) and percentage of RV fibrosis indicated same degree of cardiomyocyte enlargement and collagen deposition in both models (n=7/29 for MCT protocol; n=7/12 for PAB protocol). Results are presented as the mean $\pm$ SEM. *p<0.05, **p<0.01, ***p<0.001 vs respective control. One-way ANOVA was used for all parameters presented.

Figure S7. Signs of PAH 14 days after monocrotaline (MCT) injection. **(A)** Diagram of rat experimental MCT protocol. **(B, C)** Pulmonary flow is already compromised at this stage, with decreased pulmonary artery velocity-time integral (VTI) and the pulmonary acceleration/ejection time (PAAT/PAET) ratio. **(D, E)** RV increased afterload, as measured by pulmonary vascular resistance (PVR) is present, as well as RV end-systolic pressure (RVSP). **(F, G)** RV function is not compromised 14 days after MCT administration, without alterations in ejection fraction (EF) and cardiac output (CO). **(H-J)** Right ventricular hypertrophy is already present, as well as Lung oedema (n=6-10/group). Results are presented as the mean±SEM. *p<0.05, **p<0.01, ***p<0.001 versus C. Student t-test was used for all the parameters presented.

Figure S8. Starting chronic treatment with hUcn-2 at 14 days after MCT administration is able to stop the progression of already established pulmonary vascular remodeling. **(A, D)** In all and small arterioles, the mean Lumen/Total Ratio in MCT animals after 24 days of injection (M24D) and after 14 days (M14D) is decreased compared to CTRL rats, whereas in MU animals the Lumen/Total Ratio is significantly increased compared to M24D animals. **(B, E)** In all and small arterioles, the mean medial thickness in M24D and M14D animals is increased compared to CTRL rats, whereas in small vessels of MU animals is significantly decreased compared to M24D animals. **(C, F)** In all and small arterioles, the mean degree of vessel occlusion in M24D and M14D animals is increased compared to CTRL rats, whereas in MU animals is significantly decreased compared to M24D animals. **(G)** Representative H&E staining of lung samples of all experimental groups. Scale bars represent 50μm (400X magnification) (n=5-11/group). Results are presented as the mean±SEM. *p<0.05, **p<0.01, ***p<0.001 versus C; #p<0.05; ##p<0.01 versus M24D. One-way ANOVA was used for all parameters presented.

Figure S9. hUcn-2 administration acutely, but not chronically, stimulates the principal signalling pathways activated by CRH peptide family on the RV. **(A, B, C)** Immunoblotting shows PKA, CaMKII, and AKT phosphorylation on the RV after 0, 15, 30 and 60 minutes of hUcn-2 stimulation (i.p. injection; 5ug/kg), proving that, the dose used in each injection acts specifically on the RV (n=2/each time-point). **(D, G, E, H, F, I)** Protein phosphorylation of PKA, CaMKII, and AKT in the RV of control group non-treated (C) and treated chronically with hUcn-2 (CU; i.p. injection; 5ug/kg; bi-daily; 10 days) (n=3-9/group). Results are presented as the mean $\pm$ SEM. Student t-test was performed to compare groups in panels G, H and I.

Figure S10. Major findings of the experimental study

Supplementary References

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Control

MCT

Negative Control

A **B** **C** **D** **E** **F** **G** **H** **I** **J**