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Soluble guanylate cyclase stimulator, *trans*-4-methoxy-β-nitrostyrene, has a beneficial effect in monocrotaline-induced pulmonary arterial hypertension in rats

Karoline Gonzaga-Costa [a], Alfredo Augusto Vasconcelos-Silva [a], Matyelle Jussára Rodrigues-Silva [a], Conceição da Silva Martins Rebouça [b], Glória Pinto Duarte [c], Rosivaldo Santos Borges [d], Pedro Jorge Caldas Magalhães [a], Saad Lahlou [a,*]

[a] Department of Physiology and Pharmacology, School of Medicine, Federal University of Ceará, CE, Brazil
[b] Department of Morphology, Lab Nempi, School of Medicine, Federal University of Ceará, CE, Brazil
[c] Department of Physiology and Pharmacology, Federal University of Pernambuco, Recife, PE, Brazil
[d] Department of Pharmacy, Federal University of Pará, Belém, PA, Brazil

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ABSTRACT

The soluble guanylate cyclase (sGC)/GMPc pathway plays an important role in controlling pulmonary arterial hypertension (PAH). We investigated whether the novel sGC stimulator *trans*-4-methoxy-β-nitrostyrene (T4MN), ameliorates monocrotaline (MCT)-induced PAH. At Day 0, rats were injected with MCT (60 mg/kg, s. c.). Control (CNT) rats received an equal volume of monocrotaline vehicle only (s.c.). Four weeks later, MCT-treated rats were orally treated for 14 days with T4MN (75 mg/kg/day) (MCT-T4MN group) or its vehicle (MCT-V group), and with sildenafil (SIL; 50 mg/kg) (MCT-SIL group). Compared to the CNT group, MCT treatment induced a significant increase in both the Fulton index and RV systolic pressure but significantly reduced the maximum relaxation induced by acetylcholine. Indeed, MCT treatment increased the wall thickness of small and larger pulmonary arterioles. Oral treatment with T4MN and SIL reduced the Fulton index and RV systolic pressure compared to the MCT-V group. Maximum relaxation induced by acetylcholine was significantly enhanced in MCT-SIL group. Both T4MN and SIL significantly reduced the enhanced wall thickness of small and larger pulmonary arterioles. Treatment with T4MN has a beneficial effect on PAH by reducing RV systolic pressure and consequently right ventricular hypertrophy, and by reducing pulmonary artery remodeling. T4MN may represent a new therapeutic or complementary approach for the treatment of PAH.

1. Introduction

Pulmonary arterial hypertension (PAH) is a severe, progressive disease of small pulmonary arteries, which if left untreated, usually culminates in right heart failure and death. One of the main causes of PAH is increased pulmonary vascular resistance (PVR), which results from sustained vasoconstriction, excessive pulmonary vascular remodeling, *in situ* thrombosis, and increased pulmonary vascular stiffness (Peacock, 1999; Steiner et al., 2005). From a hemodynamic point of view, PAH corresponds to a sustained increase in mean pulmonary arterial pressure above 25 mmHg at rest compared to normal values of 10–18 mmHg for a healthy adult. However, mechanisms involved in PAH pathogenesis are complex and multifactorial. To date, three pharmacological targets have been explored in drug therapy because they interfere with endothelin-1 (ET-1), prostacyclin and nitric oxide (NO) signaling pathways (Galiè et al., 2013). This allowed development of approved drugs to inhibit the ET-1 pathway (ET-1 receptor antagonists) or to activate the prostacyclin (e.g., prostacyclin), or NO pathways (inhalation of NO, PDE5 inhibitors, and soluble guanylate cyclase (sGC) stimulators and activators).

Endothelial dysfunction is reported as an important factor in the early development of PAH (Morrell et al., 2009). The NO/sGC/cGMP signaling pathway regulates vascular tone and pulmonary resistance in PAH (Arnold et al., 1977; Evgenov et al., 2006) as well as vascular cell proliferation, platelet aggregation, and leukocyte recruitment (vascular

* Corresponding author. Department of Physiology and Pharmacology, Laboratory of Cardiovascular Pharmacology; Federal University of Ceará, School of Medicine, Rua Cel. Nunes de Melo 1315, Rodolfo Teófilo, Fortaleza, CE, 60430-270, Brazil.
E-mail addresses: lahlou562@gmail.com, saad.lahlou@ufc.br (S. Lahlou).

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remodeling), which are involved in pathogenesis of PAH (Stasch et al., 2011). To date, the sGC stimulator, riociguat (BAY 63–252), is currently the only treatment available for two types of pulmonary hypertension: idiopathic PAH and inoperable chronic thromboembolic pulmonary hypertension (Ghofrani et al., 2013; Galiè et al., 2015; Khouri et al., 2018).

Nitroderivatives found in higher plants are rare. Previously, we showed that 1-nitro-2-phenylethane (NPa), the unique nitro compound isolated from plants (Gottlieb and Magalhães, 1960), induced vasorelaxation in isolated aortic and small resistance artery preparations from both normotensive and spontaneously hypertensive rats (Interaminense et al., 2013; Brito et al., 2013). This effect involves activation of soluble guanylate cyclase (sGC)/cGMP in a nitric oxide (NO)-independent manner, resulting in increased intracellular cGMP levels (Brito et al., 2013). Conformational restriction of NPa resulted in formation of 1-nitro-2-phenylethene (NPe), a β-nitrostyrene derivative. NPe was about 3.5 times more potent as a vasorelaxant agent than its parent drug, NPa (Arruda-Barbosa et al., 2014).

In order to assess the influence of electronic structural modifications, we synthesized several new nitroderivatives in which electron donors were bonded in the aromatic ring of NPe. Little is known about the pharmacological activities of the methoxy derivative, *trans*-4-methoxy-β-nitrostyrene (T4MN; Fig. 1). Previously, we showed that T4MN displayed vasorelaxant activity in aortic rings through a mechanism similar to that described for NPa (Arruda-Barbosa et al., 2017). In the present study, we assessed whether T4MN treatment reverses PAH induced by monocrotaline (MCT) in rats. This model of MCT-induced PAH is closely mimics processes occurring (Campian et al., 2006).

2. Materials and methods

2.1. Synthesis of trans-4-methoxy-β-nitrostyrene

Synthesis of T4MN or 1-((E)-2-nitro-vinyl)-(4-methoxy)-benzene was performed at the Department of Pharmacy, Federal University of Pará, Belém, employing the Claisen–Schmitd's procedure (Vogel, 1989; Ford et al., 1994) with p-anisaldehyde and nitromethane as substrates (0.1 and 0.12 eq., respectively). The aromatic aldehyde was converted in a 'onepot' procedure, with a 96% yield, to the corresponding β-nitrostyrene by treatment with 0.05 eq. of NaOH in methanol and water at 0–10 °C (1:2) (Rosini, 1991). The resulting precipitate was filtered and dried under vacuum to yield the desired β-nitrostyrene derivative, T4MN. The trans product is preferable to the cis form due to a stereoselective reaction that gives a low energy product. T4MN was crystallized in ethanol as yellow solid-crystals, m. p. 86.6–88.2 °C (86–88 °C; Sigma-Aldrich standard). The final product was identified by NMR (^{1}H and ^{13}C NMR) and FT-IR spectroscopy and compared with properties in the literature (Wang and Wang, 2002). IR ν_{max} (cm^{-1}) 806.25, 1624.06, 1456.26, 2900, 1600, 1550, 1498, 1375; ^{1}H NMR (CD_3SOCD_3, 300 MHz) δ 7.87 (dd, 2 H), 7.52 (dd, 2 H), 7.47 (d, 1 H), 6.87 (d, 1 H), 3.76 (s, 3 H); ^{13}C NMR (CD_3SOCD_3, 75 MHz) δ 162.89, 139.12, 135.09, 131.33, 122.48, 114.87, 55.50.

2.2. Animals

Adult male Wistar rats (240–300 g) from our breeding facility were kept under conditions of constant temperature (22 ± 2 °C) with a 12 h light/12 h dark cycle. Rats had access to normal rodent diet and tap water *ad libitum*. All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals of the Brazilian National Council for Animal Experimentation. All animal experiments were reviewed and approved by the Commission for the Ethical Use of Animals (CEUA) of the Federal University of Ceará (96/2016).

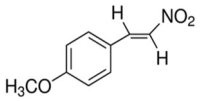

Fig. 1. Chemical structure of *trans*-4-methoxy-β-nitrostyrene.

2.3. Solutions and drugs

The perfusion medium used was a fresh modified Krebs-Henseleit solution (KHS, pH 7.4) of the following composition (in mM): NaCl 118; KCl 4.7; $NaHCO_3$ 25; $CaCl_2.2H_2O$ 2.5; KH_2PO_4 1.18; $MgSO_4.7H_2O$ 1.18; glucose 11. Salts were purchased from Merck (Darmstradt, Germany) and Vetec (Rio de Janeiro, Brazil). Phenylephrine (PHE) hydrochloride, acetylcholine (ACh) chloride, sildenafil (SIL) and MCT were purchased from Sigma. PHE and ACh were dissolved in distilled water and then with KHS to achieve desired concentration in the bath chamber. T4MN was dissolved in DMSO and ethanol, brought up to the desired concentration using saline solution, and sonicated just before use. MCT was initially dissolved in 0.5 N HCl, and the pH was adjusted to 7.4 with 0.5 N NaOH, brought up to the desired concentration using saline solution. SIL was dissolved in distilled water.

2.4. Induction of pulmonary hypertension in rats and experimental design

PAH was induced by a single subcutaneous (s.c.) injection of MCT at a dose of 60 mg/kg. Rats were randomly divided into three experimental groups (n = 5–8 per group): Control (CNT) rats (received one single s. c. Injection of MCT's vehicle at Day 0, n = 5), MCT-V rats (received MCT at Day 0 and from Day 29 after MCT injection were orally treated with T4MN's vehicle once a day for two weeks, n = 8), MCT-T4MN rats (received MCT at Day 0 and from Day 29 after MCT injection were orally treated with 75 mg/kg T4MN once a day for two weeks, n = 6), and MCT-SIL (received MCT at Day 0 and from Day 29 after MCT injection were orally treated with 50 mg/kg SIL once a day for two weeks, n = 6). SIL was used as positive control (Yoshiyuki et al., 2016). The dose of 75 mg/kg of T4MN was based upon our previous investigation regarding the anti-inflammatory activity of NPa in mice (Vale et al., 2013). Rats of all groups were examined on Day 42 of the study. In all experimental groups, body weight (BW) was determined daily throughout treatment and the dose of T4MN was adjusted accordingly.

2.5. Invasive hemodynamic measurement

Rats were anesthetized with sodium pentobarbital (60 mg/kg, i. p.). The degree of anesthesia was assessed by pinching the animal's paw with forceps and by rapid eye movements. The trachea was cannulated. For recording right ventricle systolic pressure (RVSP), a polyethylene catheter (PE-50) was inserted into the right jugular vein and advanced until large positive pulsations appeared, indicating that it was placed in the right ventricle (RV). This catheter was fixed with a suture and its position was verified by postmortem examination. RVSP was measured using a pressure transducer (Statham P23 DI) coupled to a data acquisition system (PowerLab 8/30, model ML870, ADInstruments, Bella Vista, NSW, Australia). RVSP tracings corresponding to an average of 15–20 pressure cycles were recorded and analyzed offline using Labchart Reader version 8.1.5. At the end of the hemodynamic measurement, rats were killed by exsanguination under anesthesia, and the lungs and heart were quickly removed in a block and washed in KHS. The thoracic aorta artery was removed to study vascular reactivity, and the heart was removed to assess RV hypertrophy while the lungs were removed to assess pulmonary arterial remodeling.

2.6. Vascular reactivity

This series of experiments was conducted to evaluate whether treatment with T4MN reverses endothelial dysfunction in the MCT-induced PAH model. For this purpose, the thoracic aorta was removed

and immersed in perfusion medium at room temperature. After removal of adipose tissue and adhering connective tissue, the aorta was cut into rings (approximately 2–3 mm in length) which were gently suspended with two stainless-steel hooks passed through the vessel lumen in a 5-mL organ bath containing KHS at 37 °C. They were continuously aerated with a mixture of 95% O_2 and 5% CO_2 (pH = 7.4). Isometric tension was recorded using an isometric force transducer (ML870B60, AD Instruments, Bella Vista, Australia) connected to an acquisition system (PowerLab 8/30, ML870 model, ADInstruments, Bella Vista, NSW, Australia). Tissues were equilibrated for 60 min under a resting tension of 1 g. After the equilibration period, aortic rings underwent another 1 h of stabilization during which MKHS was replaced every 15 min with fresh solution to remove metabolites. Tissues were then challenged with 75 mM KCl to confirm tissue viability. Initially, each aortic ring was contracted by exposure to 60 mM KCl and experiments were only conducted when reproducible contractions were obtained. Aortic rings were challenged with 1 μg ACh to evaluate integrity of the endothelium before any experiment. Preparations were considered to possess intact endothelium, when the vasorelaxant response to ACh was greater than 75% from the plateau of PHE-induced contraction. The relaxing effects of increasing ACh (10^{-9} to 10^{-5} M) concentrations on sustained PHE (1 μM) contractions were studied in endothelium-intact aortic rings from the three experimental groups. Data were expressed as % reversal of contraction induced by PHE.

2.7. Assessment of right ventricle hypertrophy

Right ventricular hypertrophy was assessed by determining the Fulton index (Fulton et al., 1952). During dissection of the cardiac chambers, the RV was isolated from the left ventricle (LV) and septum (S), and the latter form a single set. The RV hypertrophy index was calculated as RV/LV + S weight ratio and by the RV to BW ratio (Kaur et al., 2015). The lungs were weighted and the lung to BW ratio was also determined.

2.8. Pulmonary artery remodeling

The right and left pulmonary lobes were cut longitudinally and processed for dehydration, and embedding in paraffin blocks. Blocks were cut into 4-μm sections and stained with hematoxylin and eosin and examined using light microscopy (NIKON Eclipse E200, Japan) by an investigator who was blinded to the treatment groups to qualitatively assess vascular remodeling that occurs in the MCT-induced PAH model. Diameters of the vessels analyzed ranged between <50 μm and 50–100 μm. Sixty vessels with comparable outer diameters were randomly measured from each group. From the images scanned with ImagenJ (5.0), measurements included the outer and inner circumferences of the vessels, with areas delimited by the outer and inner elastic laminae, respectively. Vessel diameter was calculated from the medial circumference, and the vascular wall area was calculated by subtracting the luminal circumference from the outer medial circumference. Wall thickness was expressed as a percentage of the total vessel area, according to the following formula: [(outer area) - (inner area)/outer area]/100.

2.9. Statistical analysis

Results are expressed as means ± S.E.M.. Survival analysis was performed using Kaplan-Meier survival analysis. Pairwise comparisons between survival groups were performed using the Log-rank test. IC_{50} values, defined as the concentration (μM) of ACh required to produce a half-maximal reduction of the PHE-induced contractile response, were calculated by interpolation from semi-logarithmic plots. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). Significance ($P < 0.05$) of results was assessed by paired Student's *t*-test, Mann-Whitney *U* test, and one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test as appropriate.

3. Results

3.1. Rat survival

Survival curves of the four experimental groups are shown in Fig. 2. It is noteworthy that, in the MCT-T4MN group, two rats died at the end of 42 days of follow-up which may indicate increased survival time. However, comparison of the survival rate of MCT-V with respect to MCT-T4MN and MCT-SIL groups using the Logrank test did not reveal a significant difference among the groups ($P > 0.05$).

3.2. Morphometric data

As expected, RV weight to BW ratio (Fig. 3A) and Fulton index (Fig. 3B), that is the right ventricle to left ventricle plus septum ratio, were significantly increased in the MCT group compared to CNT rats ($P < 0.001$), indicating the existence of RV hypertrophy. Both these measured parameters were significantly reduced by oral treatment with T4MN and SIL (Fig. 3; $P < 0.05$). The significant increase (Fig. 4; $P < 0.01$) of lung to BW ratio in the MCT group compared to CNT group indicates the development of pulmonary inflammation, an effect that was abolished in the MCT-SIL group (Fig. 4; $P < 0.05$), but not in the MCT-T4MN group (Fig. 4; $P > 0.05$).

3.3. Hemodynamic parameters

RVSP was fourfold higher in MCT-V group (Fig. 5; $P < 0.001$) than that recorded in the CNT group, indicating the presence of very severe PAH. Oral treatment with T4MN or SIL for 14 days significantly reduced ($P < 0.01$) the enhanced RVSP (Fig. 5A and B).

3.4. Reactivity vascular data

In endothelium-intact aortic rings from the CNT group, increasing concentrations of ACh induced concentration-dependent vasorelaxation with an IC_{50} of 1.07 ± 0.33 μM (Fig. 6). This vasorelaxation was attenuated in the MCT-V group as evidenced by the enhanced IC_{50} value of ACh-induced vasodilation (>10 μM). Maximal relaxation responses from all experimental groups are shown in Table 1. Oral treatment with SIL, restored the IC_{50} (0.66 ± 0.21 μM) (Fig. 6) as well as the maximal

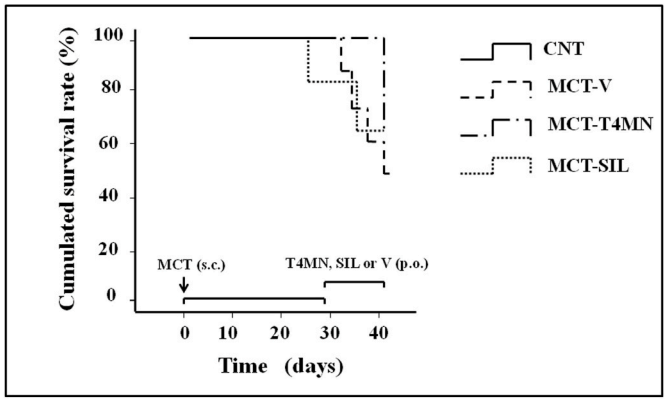

Fig. 2. Treatment with *trans*-4-methoxy-β-nitrostyrene failed to enhance survival rate. Survival rate was significantly decreased in MCT-injected animals treated with vehicle (MCT-V) or with sildenafil (MCT-SIL). In rats injected with MCT and treated orally with *trans*-4-methoxy-β-nitrostyrene (T4MN; 75 mg/kg/day; MCT-T4MN group), two rats died on the last day (Day 42) of MCT treatment. No rats in the control (CNT) group died. MCT: monocrotaline; s. c.: subcutaneous; V; vehicle.

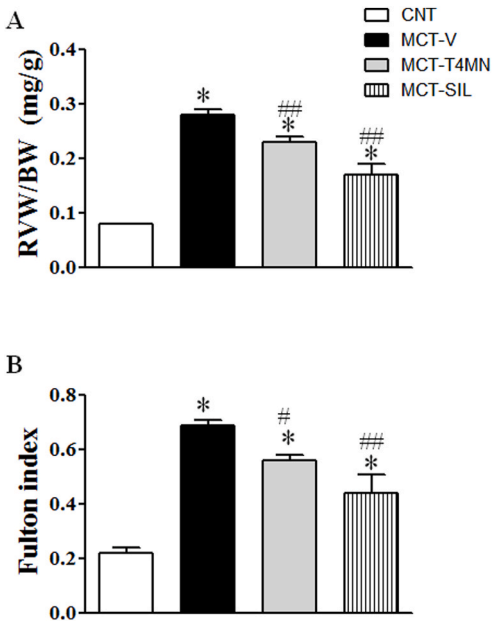

Fig. 3. Treatment with *trans*-4-methoxy-β-nitrostyrene reduced right ventricle hypertrophy. Morphometric data collected in control (CNT) rats, those injected only with monocrotaline (MCT; 60 mg/kg, s. c.) (MCT-V group) and those injected with MCT and treated orally with *trans*-4-methoxy-β-nitrostyrene (T4MN; 75 mg/kg/day) (MCT-T4MN group) or sildenafil (SIL; 50 mg/kg/day) (MCT-SIL group) during 14 the days from Day 29 after MCT injection. In **A**, right ventricle weight (RVW) to body weight (BW) ratio. In **B**, Fulton index, i.e. the right ventricle to left ventricle plus septum weight ratio. Data are expressed as means S.E.M. (n = 4–5 rats per group). $^{*}P < 0.001$, with respect to CNT group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ with respect to MCT-V group, by one-way ANOVA followed by Dunnett's test..

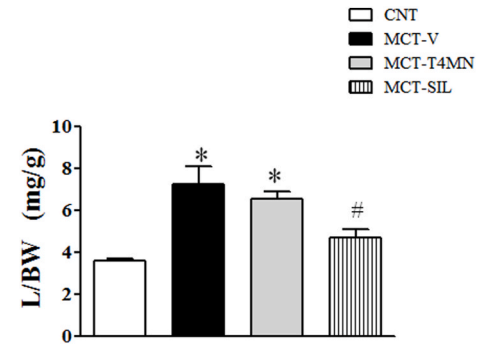

Fig. 4. Treatment with *trans*-4-methoxy-β-nitrostyrene failed to alter lung hypertrophy. Lung to body weight ratio in control (CNT) rats, those injected only with monocrotaline (MCT; 60 mg/kg, s. c.) (MCT-V group) and those injected with MCT and treated orally with *trans*-4-methoxy-β-nitrostyrene (T4MN; 75 mg/kg/day) (MCT-T4MN group) or sildenafil (SIL; 50 mg/kg/day) (MCT-SIL group) during 14 days from the Day 29 after MCT injection. Data are expressed as means S.E.M. (n = 4–5 rats per group). $^{*}P < 0.05$, $^{**}P < 0.01$ with respect to CNT group; $^{\#}P < 0.01$ with respect to MCT-V group, by one-way ANOVA followed by Dunnett's test.

relaxation response to ACh (Table 1). However, oral treatment with T4MN failed to reverse the endothelial dysfunction (Fig. 6) as the enhanced maximal ACh relaxation evoked by T4MN did not reach statistical significance (Table 1).

3.5. Vascular morphology of pulmonary arterioles

Fig. 7A and B provide representative images of pulmonary terminal arterioles in the four experimental groups. In pulmonary arterioles with an external diameter <50 μm, wall thickness was significantly (Fig 7C; $P < 0.001$) increased in the MCT-V group compared with CNT rats, indicating MCT-induced pulmonary vascular remodeling. Oral treatment with T4MN and SIL significantly reduced ($P < 0.001$) the enhanced wall thickness of these vessels. In larger pulmonary arterioles (50–100 μm) from MCT-injected rats, wall thickness was also enhanced (Fig 7D; $P < 0.001$), an effect that was also significantly reduced by T4MN and SIL treatment (Fig. 7D; $P < 0.01$).

4. Discussion

In MCT-T4MN, survival time was increased with respect to MCT-V and MCT-SIL groups (Fig. 2). However, comparison of survival rate of MCT-V with respect to MCT-T4MN and MCT-SIL groups by means of the Logrank test did not reveal a significant difference among the groups. The apparent increased survival would have reflected beneficial effects of T4MN treatment on cardiac and pulmonary vessel remodeling, and ventricular hypertrophy as well as on increased RVSP. Given the vasodilatory properties of T4MN through stimulation of the sGC/cGMP pathway by the vascular endothelium (Arruda-Barbosa et al., 2017), we investigated whether treatment with T4MN could normalize hemodynamic changes and cardiac hypertrophy in this model of PAH. In our experimental conditions, chronic treatment with T4MN for 14 days after the establishment of PAH was able to prevent RV hypertrophy, reduce pulmonary vascular remodeling, and improve RV function in animals with PAH.

Experimental models of PAH are widely used to study and understand the pathophysiological mechanisms of this disease, and the use of monocrotaline (MCT) for its induction is a well-established model. MCT induces injury to pulmonary capillaries with increased PVR and ventricular afterload, progressively causing pathological remodeling of the RV with the induction of hypertrophy, increased interstitial fibrosis, endothelial dysfunction, and heart failure (Pacagnelli et al., 2016). The experimental model of MCT-induced PAH employed in the current study is valid and could be comparable to the clinical situation since, compared to the CNT group, animals from the MCT-V group had right ventricular hypertrophy, pulmonary edema, increased RVSP, pulmonary vascular remodeling, and endothelial dysfunction.

Compared to the CNT group, animals treated with MCT showed an increased Fulton index and RV to BW ratio, indicating development of RV hypertrophy that may worsen RV systolic function. Oral T4MN, as well as SIL, treatment reduces pathological MCT-induced cardiac ventricular remodeling since the RV to BW ratio and Fulton index in the MCT-T4MN and MCT-SIL groups were statistically reduced compared to those recorded in MCT-V group. Regarding pulmonary edema indicators, initial inflammation and alveolar damage followed later by fibrosis and pulmonary vascular remodeling are changes of MCT-promoted pulmonary histopathological findings, which within twenty-one days, lead to an increase in capillary permeability, resulting in interstitial and alveolar edema (Dumitrascu et al., 2008). Compared with the CNT group, the lung to BW ratio in the MCT-V group was significantly increased, as was expected. A 14-day SIL treatment significantly reduced the MCT-induced increase in lung to BW ratio, an effect that was not observed in PAH rats treated with T4MN.

Endothelial dysfunction is reported as an important factor in the early development of PAH (Morrell et al., 2009). In the present study, evaluation of endothelial dysfunction was performed using thoracic aorta although a distinct tissue sensitivity to MCT (aorta vs. pulmonary aorta) has been reported (Gout et al., 1999). MCT significantly impaired ACH-induced aortic preparations evidenced by a significant right shift of the concentration-response curve and a reduction of E_{max} and increased IC_{50} compared with the control group. This is in agreement with previous studies using thoracic aorta in PAH (Zapata-Sudo et al., 2012; Steven et al., 2017). Sildenafil treatment significantly improved the endothelial function; however, T4MN treatment failed to improve the

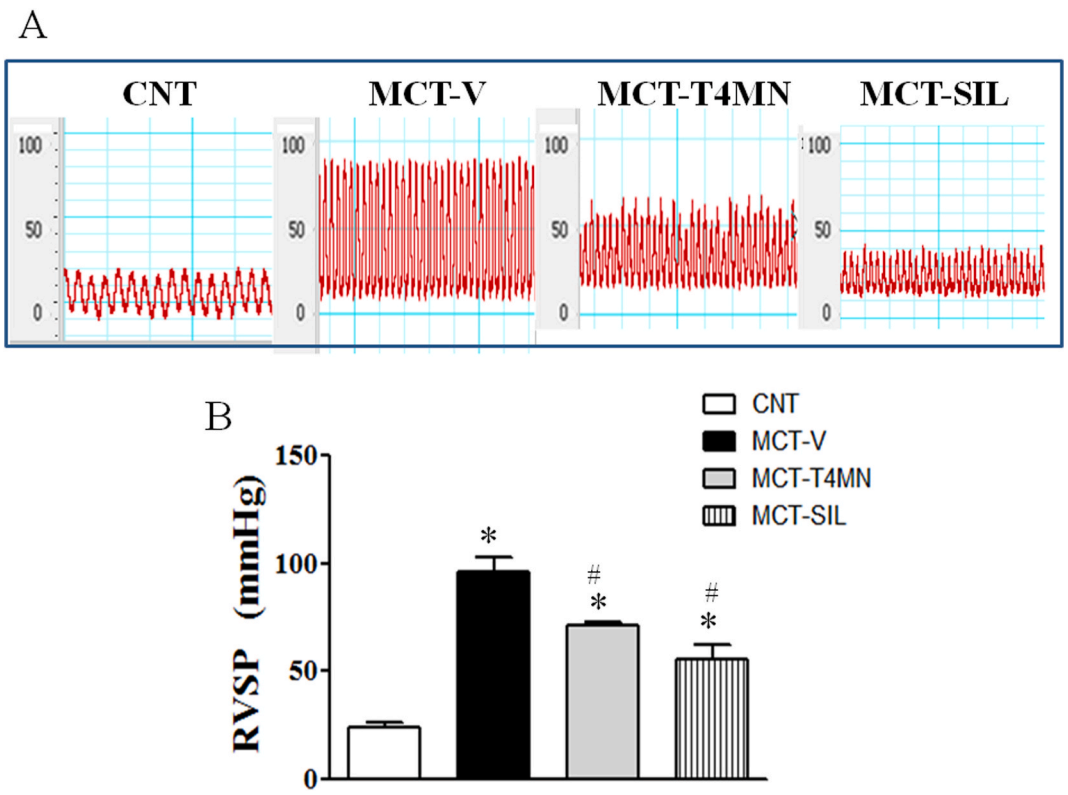

Fig. 5. Treatment with 1 *trans*-4-methoxy-β-nitrostyrene reduced the monocrotaline-induced increased right ventricle systolic pressure. In **A**, representative tracings of right ventricle systolic pressure (RVSP) in control (CNT) rats, and in rats with monocrotaline (MCT)-induced pulmonary arterial hypertension that had been treated orally for two weeks following Day 29 after MCT injection with *trans*-4-methoxy-β-nitrostyrene (T4MN; 75 mg/kg/day) (MCT-T4MN group) or its vehicle (MCT-V group), or with sildenafil (SIL; 50 mg/kg/day) (MCT-SIL). In **B**, RVSP was fourfold higher than in the CNT group, indicating severe PAH. Oral treatment with T4MN and SIL reduced the MCT-induced increased RVSP. Data are expressed as means ± S.E.M. (n = 4–5 rats per group). $^{**}P < 0.001$ with respect to the CNT group; $^{\#}P < 0.01$ with respect to MCT-V group by one-way ANOVA followed by Dunnett's test.

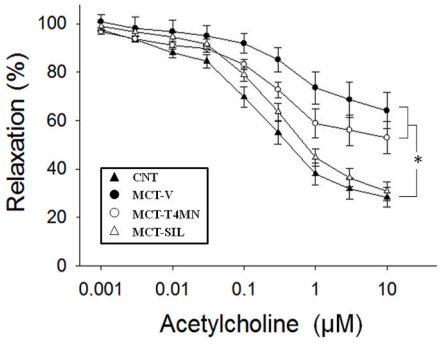

Fig. 6. Treatment with *trans*-4-methoxy-β-nitrostyrene failed to improve endothelial function. Concentration-response curves for relaxation induced by acetylcholine from control (CNT) rats, and in rats with monocrotaline (MCT)-induced pulmonary arterial hypertension that had been treated orally for two weeks following Day 29 after MCT injection with *trans*-4-methoxy-β-nitrostyrene (T4MN; 75 mg/kg/day) (MCT-T4MN group) or its vehicle (MCT-V group), or with sildenafil (SIL; 50 mg/kg/day) (MCT-SIL). Data are expressed as means ± S.E.M. (n = 9–14 per group). $^{*}P < 0.01$ *vs.* MCT group by two-way ANOVA.

endothelial function and vascular tone as its enhancement of the reduced E_{max} induced by MCT was insignificant. Nevertheless, protection offered by T4MN against PAH may result from vasodilatation, which reducing pulmonary vascular resistance. The NO/sGC/cGMP signaling pathway regulates vascular tone and pulmonary resistance in PAH (Arnold et al., 1977; Evgenov et al., 2006) as well as vascular cell proliferation, platelet aggregation, and leukocyte recruitment (vascular remodeling), which are involved in pathogenesis of PAH (Stasch et al., 2011). In our previous investigation performed in aortic preparations from normotensive rats, we showed that T4MN induced potent vasorelaxation involving stimulation of sGC/cGMP pathway, leading to increased cGMP levels (Arruda-Barbosa et al., 2017). The actions of cGMP are attributed to activation of protein kinase G (PKG), which can modulate different target effectors in vascular smooth muscle. Although activation of PKG by cAMP requires nearly a 10-fold-higher cAMP concentration than cGMP concentration, it is unlikely that it contributes to the mediation of T4MN-induced vasorelaxation since this effect was unaltered by the adenylate cyclase inhibitor MDL-12,330 A

Table 1
Oral treatment with *trans*-4-methoxy-β-nitrostyrene failed to reverse the endothelial dysfunction of aortic rings from monocrotaline (MCT)-treated rats. IC_{50} and maximum vasorelaxation (Rmax) to acetylcholine (10^{-9} to 10^{-5} M) in aortic rings from control (CNT) rats and those with pulmonary arterial hypertension (PAH) treated (MCT-T4MN) or not (MCT-V) with *trans*-4-methoxy-β-nitrostyrene (T4MN) or those with PAH treated with sildenafil (MCT-SIL) used as positive control.

Acetylcholine		
Groups	IC_{50} (μM)	Rmax (%)
CNT	1.07 ± 0.33	71.63 ± 4.08
MCT-V	>10	35.92 ± 7.45[a]
MCT-T4MN	>10	47.05 ± 6.60[a]
MCT-SIL	0.66 ± 0.21	69.19 ± 3.89[b]

All values are means ± SEM (n = 9–14 per group). $^{a}P < 0.00$ by Mann-Whitney *U* test with respect to CNT. $^{b}P < 0.01$ by Mann-Whitney *U* test with respect to MCT-V.

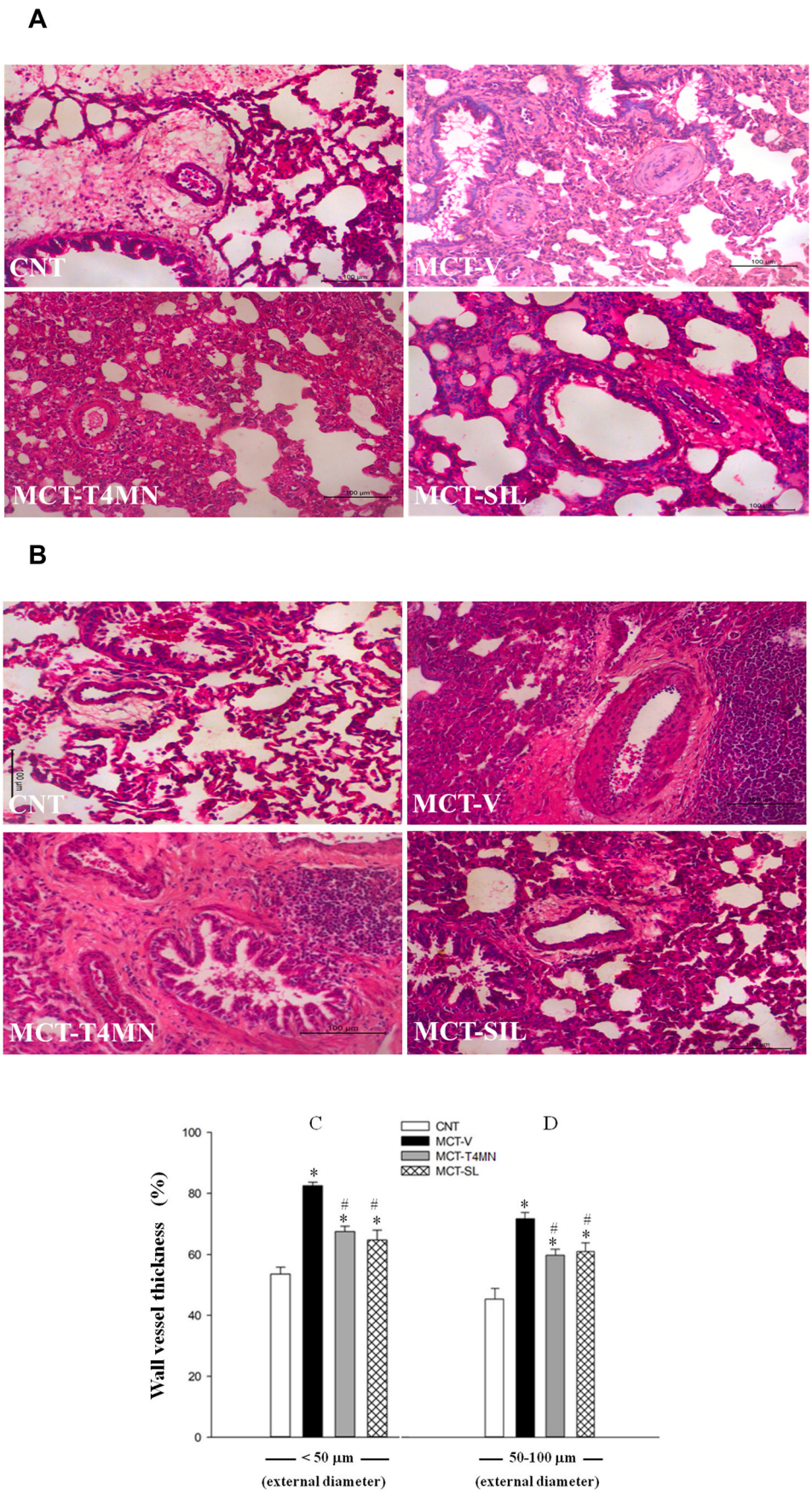

Fig. 7. Treatment with *trans*-4-methoxy-β-nitrostyrene reduces pulmonary vascular remodeling in monocrotaline-induced PAH. In **A and B**, Representative images of lung sections stained with hematoxylin and eosin in three different groups: control (CNT) rats, monocrotaline (MCT)-injected rats treated with vehicle (MCT-V), MCT-injected rats treated with *trans*-4-methoxy-β-nitrostyrene (MCT-T4MN), or MCT-injected rats treated with sildenafil (MCT-SIL). In **A**, pulmonary arterioles with an external diameter <50 μM. In **B**, pulmonary arterioles with an external diameter between 50 and 100 μM. Images show vessels at 20× magnification. In **C**, wall thickness of vessels with an external diameter <50 μM. In **D**, wall thickness of vessels with an external diameter between 50 and 100 μM. Wall thickness was expressed as a percentage of total area. All data are means ± S.E.M. (n = 60 images per group) $^{*}P < 0.001$ *vs.* CNT; $^{\#\#}P < 0.001$ *vs.* MCT group by one-way ANOVA followed by Dunnett's test.

(Arruda-Barbosa et al., 2017). In aortic rings, T4MN-activated PKG mediates opening of potassium channels and reduction of intracellular Ca^{2+} by partial inhibition of Ca^{2+} influx through either voltage-operated calcium channels or intracellular Ca^{2+} stores. Such a mode of action is consistent with the ability of T4MN to stimulate sGC. This stimulation was also cridited for the vasorelaxant effects of T4MN in resistance mesenteric artery preparations from spontaneously hypertensive rats (Alves-Santos et al., 2019). Recently, we showed that T4MN evokes endothelium-independent vasorelaxant effects in isolated rat pulmonary artery, partially by inhibiting Ca^{2+} influx through L-type Ca^{2+} channels, as well as by activating sGC and potassium channels (Arruda-Barbosa, 2021). Notably, the IC_{50} for vasorelaxant effects of T4MN in endothelium-intact aortic rings pre-contracted with phenylephrine from normotensive rats was about 57.64 μM (Arruda-Barbosa et al., 2017), a value that was of the same order of magnitude as that (48.60 μM) recorded for vasorelaxant effects of T4MN in pulmonary artery rings (Arruda-Barbosa, 2021). Although this observation may suggest that the pulmonary artery is no more sensitive than aorta, this sGC stimulator, T4MN, could be a promising candidate not only as an antihypertensive drug, but also for treatment of PAH.

MCT injection in rats results in enhanced RVSP and pulmonary vessel wall thickness and PAH. Our results showed that after 14-day period treatment, T4MN reduced the enhanced RVSP, as with the positive control, SIL. T4MN reduced pulmonary vascular remodeling as evidenced by the decreased vessel wall thickness of small (<50 μm) and larger (50–100 μm) pulmonary arterioles, as corroborated with hemodynamic data. At the present time, we have no clues regarding the exact mechanism by which T4MN treatment prevents pulmonary vascular remodeling. Further experiments are necessary to address this issue. As far as we know, this is the first study to report beneficial effects of T4MN on hemodynamic changes and pulmonary vascular remodeling and therefore, PAH.

Our study has some limitations. The first is that the question of whether T4MN treatment inhibited the progression of PAH or had regressive effects cannot be answered by findings of the present study. Further experiments to be performed 29 days after MCT injection are needed to assess this issue. Furthermore, selection of only an MCT-induced animal model for PAH may be another limitation of this study in the context of clinical PAH.

5. Conclusions

Taken together, our data suggest that treatment with T4MN has beneficial effect on PAH by reducing RVSP and consequently right ventricular hypertrophy, and by reducing pulmonary vascular remodeling. It is suggested that T4MN may represent a new therapeutic or complementary approach to the treatment of PAH.

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CRediT authorship contribution statement

Karoline Gonzaga-Costa: performed experiments regarding to induction of pulmonary hypertension, assessment of right ventricle hypertrophy, pulmonary artery remodeling, hemodynamic measurement and statistical analysis of data; review & editing of the manuscript. **Alfredo Augusto Vasconcelos-Silva:** performed vascular reativity experiments, statistical analysis of data. **Matyelle Jussára Rodrigues-Silva:** contributed to experiments of hemodynamic measurement, statistical analysis of data. **Conceição da Silva Martins Rebouça:** contributed to assessment of pulmonary artery remodeling, statistical analysis of data. **Glória Pinto Duarte:** Writing - review & editing, Funding acquisition, contributed to funding aquisition, review & editing of the manuscript. **Rosivaldo Santos Borges:** performed synthesis of the nitroderivatives, contributed to funding aquisition, review & editing of the manuscript. **Pedro Jorge Caldas Magalhães:** Writing - review & editing, Supervision, review & editing of the manuscript. **Saad Lahlou:** Supervision, Conceptualization, "Writing - original draft, contributed to funding aquisition, and review & editing.

Declaration of competing interest

The authors declare that they no conflict of interest regarding publication of this article.

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