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Apelin decreases myocardial injury and improves right ventricular function in monocrotaline-induced pulmonary hypertension

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Falcão-Pires I, Gonçalves N, Henriques-Coelho T, Moreira-Gonçalves D, Roncon-Albuquerque R Jr, Leite-Moreira AF. Apelin decreases myocardial injury and improves right ventricular function in monocrotaline-induced pulmonary hypertension. *Am J Physiol Heart Circ Physiol* 296: H2007–H2014, 2009. First published April 3, 2009; doi:10.1152/ajpheart.00089.2009.—We investigated the endogenous production of apelin and the cardiac and pulmonary effects of its chronic administration in monocrotaline (MCT)-induced pulmonary hypertension (PH). Male Wistar rats were injected with MCT (60 mg/kg sc) or vehicle (*day 0*). One week later, these animals were randomly treated during 17 days with pyroglutamylated apelin-13 (Pyr-AP13; 200 $\mu g \cdot kg^{-1} \cdot day^{-1}$ ip) or a similar volume of saline, resulting in four groups: sham ($n = 11$), sham-AP ($n = 11$), MCT ($n = 16$), and MCT-AP ($n = 13$). On *day 25*, right ventricular (RV) and left ventricular (LV) hemodynamic and morphometric parameters were assessed. Tissue and plasma samples were collected for histological and molecular analysis. When compared with sham, the MCT group presented a significant increase of RV mass (166 ± 38%), diameter of cardiomyocyte (40 ± 10%), myocardial fibrosis (95 ± 20%), peak systolic pressure (99 ± 22%), peak rate of ventricular pressure rise (dP/dt_{max}; 74 ± 24%), peak rate of ventricular pressure decline (dP/dt_{min}; 73 ± 19%), and time constant τ (55 ± 16%). In these animals, RV expression of apelin (−73 ± 10%) and its receptor APJ (−61 ± 20%) was downregulated, whereas mRNA expression of type B natriuretic peptide (9,606 ± 713%), angiotensinogen (191 ± 147%), endothelin-1 (RV, 497 ± 156%; and LV, 799 ± 309%), plasmatic levels of apelin (104 ± 48%), and angiotensin 1-7 (161 ± 151%) were increased. Chronic treatment with Pyr-AP13 significantly attenuated or normalized these changes, preventing apelin-APJ mRNA downregulation and PH-induced neurohumoral activation of several vasoconstrictors, which exacerbates apelin-APJ vasodilator effects. Therefore, apelin delayed the progression of RV hypertrophy and diastolic dysfunction. Together, these observations suggest that the apelin-APJ system may play an important role in the pathophysiology of PH, representing a potential therapeutic target since it significantly attenuates RV overload and PH-induced neurohumoral activation.

heart failure; angiotensin receptor like-1; right ventricle hypertrophy

ANGIOTENSIN RECEPTOR like-1 (APJ) and its endogenous ligand, apelin, represent a recently discovered system that regulates cardiac function (38). Both are distributed in the heart, lungs, central nervous system, and gastrointestinal tract. In the cardiovascular system, APJ is located in cardiomyocytes and vascular smooth muscle cells, whereas apelin is produced in endocardial endothelial cells, prompting the suggestion that endothelial apelin may act as a paracrine mediator of APJ receptor to influence cardiac contractility or vascular tone (23, 25). Little is known about the regulation of apelin production in cardiovascular tissues; however, angiotensin converting enzyme-2 (ACE2), a zinc metalloproteinase that catalyzes the conversion of angiotensin II (ANG II) to angiotensin-(1–7) [ANG-(1–7)], has been identified as the only enzyme that breaks down apelin peptides (41).

The intense research on apelin has shown its involvement in the regulation of cardiovascular function (3, 10, 35), hemodynamic homeostasis (6), immune response (18), brain signaling (11, 30), and HIV infection (5), as well as insulin secretion in mice (34). Particularly, in the cardiovascular system, the apelin-APJ system has been the focus of several studies, including the human heart (7), but its physiological role is not yet completely understood. Apelin-APJ vascular effects are controversial, although suggesting that they may depend on endothelial integrity, i.e., vasodilator through endothelial-dependent nitric oxide (NO) release (8, 39), or vasoconstrictor, in the presence of a dysfunctional endothelium, by direct binding to APJ in vascular smooth muscle cells (22). In isolated rat hearts, apelin acts as one of the most potent positive inotropic substances identified to date, both in normal (1, 3, 10, 35) and in heart failure (3, 10, 12) rats, including right ventricular (RV) failure secondary to hypoxia-induced pulmonary hypertension (PH). In normal hearts, these effects depend on phospholipase C, protein kinase C, Na^+-H^+ exchanger, and Na^+-Ca^{2+} exchanger (36) activation, whereas in the failing RV due to PH it is dependent of increased calcium transients, rather than of changes in myofilament calcium responsiveness (10).

PH is a progressive disease associated with RV hypertrophy that commonly progresses to heart failure. Endothelin (ET)-1 and several other neurohumoral agents notably worsen the complex pathophysiology of PH (16, 32), and its antagonists are used as therapeutic targets for this entity. So far, the effects of apelin in PH have never been studied, even if its unusual combination of positive inotropic and afterload reduction effects suggests that the apelin-APJ pathway may represent a potential therapeutic target in PH. Furthermore, considering that apelin-APJ are abundant in heart and lungs and that its localization might provide a clue to its physiological role, we investigated whether exogenous pyroglutamylated apelin-13 (Pyr-AP13) could chronically change the pathophysiological progression of PH as well as RV functional performance. Therefore, the aim of the present study was to better understand the physiological role of the apelin-APJ system in PH and to elucidate the effects of its exogenous administration in biventricular function, pulmonary and RV structure, and neurohumoral activation as well as apelin and APJ expression and production in healthy and monocrotaline (MCT)-PH rats.

MATERIAL AND METHODS

Experimental design. Animal experiments were performed according to the Portuguese law for animal welfare and accordingly to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, Revised

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1996). The Faculty of Medicine at the University of Porto is a governmental institution granted approval by the Portuguese government to perform animal experiments as described in this study. Adult male Wistar rats (Charles River Laboratories, Barcelona, Spain) weighing 160–180 g were housed in groups of five rats per cage in a controlled environment under a 12-h:12-h light-dark cycle at a room temperature of 22°C, with a free supply of food and water.

Rats randomly received a subcutaneous injection of MCT ($n = 29$; 60 mg/kg body wt; Sigma, Barcelona, Spain) or an equal volume of vehicle ($n = 22$; 2 ml/kg body wt). One week after MCT/vehicle injection (*day 0*), 13 random MCT animals and 11 random vehicle animals received an intraperitoneal injection of Pyr-AP13 (200 $\mu g \cdot kg^{-1} \cdot day^{-1}$; Bachem, Weil and Rhein), whereas the remaining rats received equal volume of saline until *day 24*, one day before hemodynamic assessments. This chronic protocol resulted in four groups: control rats treated with saline (sham; $n = 11$) or Pyr-AP13 (sham-AP; $n = 11$) and MCT-treated rats with saline (MCT; $n = 16$) or Pyr-AP13 (MCT-AP; $n = 13$). The physiologically active form is apelin-36, but the most biologically effective are apelin-16 and the pyroglutamylated form of apelin-13. This common posttranslational modification preserves biological activity by rendering the peptide more resistant to enzymatic cleavage. Therefore, we used Pyr-AP13 for this chronic study. The peptide was dissolved in distilled, autoclaved water and frozen at −20°C.

Hemodynamic studies. Twenty-five days after MCT/vehicle injection, the rats were anesthetized by inhalation of a mixture of sevoflurane (4%) and oxygen, intubated for mechanical ventilation, and placed over a heating pad. Under binocular surgical microscopy (Wild M651.MS-D; Leica), the right jugular vein was cannulated for fluid administration (prewarmed 0.9% NaCl solution) to compensate for perioperative losses. The heart was exposed through a median sternotomy, and the pericardium was widely opened. RV and left ventricular (LV) pressures were measured with a 2F high-fidelity micromanometer (SPR-324; Millar Instruments) inserted into the RV and LV cavities, respectively. After complete instrumentation, the animal preparation was allowed to stabilize for 15 min. Hemodynamic recordings were made with respiration suspended at end expiration. Parameters were converted online to digital data with a sampling frequency of 1,000 Hz. RV and LV pressures were measured at end diastole and peak systole (P_{max}). Peak rates of RV and LV pressure rise (dP/dt_{max}) and pressure decline (dP/dt_{min}) were measured as well. The relaxation rate was estimated with the time constant τ by fitting the isovolumetric pressure fall to a monoexponential function. At the end of the experimental protocol, a blood sample (2 to 3 ml) was collected using a syringe with heparin for plasmatic quantification of apelin and ANG-(1–7).

Morphometric analysis and tissue preparation. Once hemodynamic data collection was completed, the heart, lungs, and right gastrocnemius muscle were excised and weighed. The right tibia was also excised and measured using a millimetric ruler. Under binocular magnification (×3.5), the RV free wall was dissected from the LV and weighed separately. Heart, lung, RV, and LV + septal (LV + S) weights were normalized to body weight (BW), whereas gastrocnemius weight was normalized to tibial length. Additionally, RV weight was normalized to that of LV + S.

Histology. RV and right lung samples were immersion fixed in 10% buffered formalin and embedded in paraffin. Sections 4 μm thick were cut and stained with hematoxylin and eosin. RV free wall specimens were obtained from each heart at midway between the apex and base. Studied samples were photographed with a digital camera, and the cardiomyocyte diameter was measured, in each section, using a digital image analyzer (Leica IM-1000). These measurements were made directly at ×400 magnification only in muscle fibers in which the cross section included a round nucleus. Five sections per sample were photographed, and the diameter of 10 muscle fibers was measured. The diameter of 50 analyzed cardiomyocytes per sample was then averaged. Subsequent image analysis with Slidebook 4.0 software (3I) was performed to determine the extent of myocardial reactive interstitial fibrosis (percentage). Areas of reparative and perivascular fibrosis were excluded.

Pulmonary specimens of each animal were collected from the upper right lobe. External diameter and medial wall thickness in muscular arteries (12 arteries/lung) were analyzed at ×400 magnification. Orthogonal intercepts generated eight random measurements of external diameter of the vessels (distance between the external lamina) and 16 random measurements of medial thickness of the vessels (distance between the internal and external lamina). For each artery, medial hypertrophy was expressed as follows: %wall thickness = [(medial thickness × 2)/(external diameter)] × 100.

Plasma analysis. Venous blood samples collected in ethylenediamine-tetra-acetic acid-containing tubes were spun at 5,000 rpm for 15 min at 4°C, and plasma was extracted and frozen at −70°C until analysis. Apelin assays were performed using the apelin-36 microplate ELISA assay kit (Phoenix Pharmaceuticals, Karlsruhe, Germany) according to the manufacturer's instructions. The antibody used in this apelin assay cross reacts 100% with rat apelin-36, which is known to be the precursor of all biologically active peptides of apelin. Additionally, to indirectly assess ACE2 activity, we measured the formation of its breakdown product, ANG-(1–7), in plasma samples. ANG-(1–7) assays were performed using a 96-microplate ELISA assay kit (Peninsula Laboratory, San Carlos, CA) according to the manufacturer's instructions. All plasma samples were analyzed in duplicate using an ELISA plate reader (Perkin-Elmer, Wellesley, MA). Values were normalized to a standard curve.

mRNA quantification. Two-step real-time RT-PCR was performed as previously described (31). Briefly, after total mRNA extraction (no. 74124; Qiagen), standard curves were obtained for each gene correlating ($R > 0.98$) the mRNA quantities in graded dilutions from a randomly selected tissue sample with the respective threshold cycles (second derivative maximum method). Equal amounts of mRNA from every sample underwent two-step real-time RT-PCR experiments for each gene, using SYBR green as marker (no. 204143; Qiagen). GAPDH was used as internal control because its mRNA levels were similar in the studied groups. Results are relative to the mean obtained for the sham group (set as arbitrary unit) and normalized for GAPDH. Specific PCR primer pairs for the studied genes were: GAPDH, forward 5′-TGGCCTTCCGTGTTCCTACCC-3′ and reverse 5′-CCGCCTGCTTCACCACCTTCT-3′; APJ, forward 5′-CCAGTCTGAATGTGACTACGC-3′ and reverse 5′-ACCACAAAGGTC AAGTCAGCC-3′; apelin, forward 5′-TTGACTGCCGTGTGTGGAGTGCCA-3′ and reverse 5′-AAAGGCATGGGTCCCTTATGG- 3′; type B natriuretic peptide (BNP), forward 5′-GGACCAAGGCCCTACAAAAGA-3′ and reverse 5′-CAGAGCTGGGGAAAGAAGAG-3′; ET-1, forward 5′-CCATGCAGAAAGGCGTAAAAG-3′ and reverse 5′-CGGGGCTCTGTAGTCAATGTG-3′; angiotensinogen, forward 5′-CGGACAGCACCCTATTTTTCAACA-3′ and reverse 5′- GAGGCGCACTGGGGCTGGAT-3′; Mas receptor, forward 5′-CCATCCTCAGCTTCTTGGTC-3′ and reverse 5′-GCATGGCAAAGATGAGGAAT-3′.

Statistical analysis. Statistical analysis was performed using Prim (version 5.0). Group data are presented as means ± SD and were compared using two-way ANOVA. When the normality test failed, the two-way ANOVA was preceded by a logarithmic transform to obtain a normal distribution. When treatments were significantly different, the Student-Newman-Keuls test was selected to perform pairwise multiple comparisons. Mortality rates were compared with Fisher exact test. Corelations between two continuous variables were assessed with linear regression analysis. Statistical significance was set at $P < 0.05$.

RESULTS

Somatic and cardiac growth. Data related to somatic and cardiac growth are summarized in Table 1. In the MCT group, RV, heart, and lung/BW were significantly increased. Treat-

Table 1. *Effect of chronic administration of 17-day apelin on somatic and cardiac growth*

	Sham	Sham-AP	MCT	MCT-AP
n	11	11	12	10
Body weight, g	322±35	324±34	273±24*†	283±27*†
Heart weight/body weight, g/kg	2.87±0.35	2.98±0.23	4.20±0.24*†	3.96±0.27*†
RV weight/body weight, g/kg	0.47±0.04	0.51±0.07	1.25±0.18*†	0.84±0.40*†‡§
RV/LV + S, g/g	0.24±0.02	0.25±0.04	0.52±0.05*†	0.41±0.18*†‡
Lung weight/body weight, g/kg	4.05±0.55	4.40±1.13	8.66±1.95*†	6.37±1.03*†‡§
Gastrocnemius weight/tibial length, mg/mm	47.9±7.0	50.3±3.6	43.8±4.4	43.0±4.4

Values are means ± SD; *n*, number in each group. RV, right ventricle; LV + S, left ventricle and septum; AP, apelin; MCT, monocrotaline-treated rats. Comparisons were performed using two-way ANOVA followed by the Student-Newman-Keuls test to perform pairwise multiple comparisons. *$P < 0.05$ vs. Sham; †$P < 0.05$ vs. Sham-AP; ‡$P < 0.05$ vs. MCT; §significant interaction.

ment with Pyr-AP13 attenuated the effects of MCT on RV and lung/BW, whereas no changes were observed in the sham-AP group. Interaction analysis revealed that effects of Pyr-AP13 on RV and lung/BW were significantly different in sham and MCT rats. The BW of MCT and MCT-AP rats was similar, but both were significantly lower than sham and sham-AP animals.

Twenty-five days after injection, the mortality was null in sham and sham-AP, 33% in MCT and 23% in MCT-AP groups. Although there was a trend for reduction in mortality rate in apelin-treated MCT rats compared with MCT, it did not reach statistical significance.

Hemodynamic assessment. We administrated Pyr-AP13 over the course of 17 days at a level previously shown to exert acute and chronic hemodynamic effects (21). Chronic administration of apelin significantly changed several hemodynamic parameters, especially those related to RV function (Table 2). Peak systolic RV pressure (RV P_{max}), which was used to estimate PH, RV dP/dt_{max}, dP/dt_{min}, and τ were significantly increased in the MCT group. Chronic treatment with Pyr-AP13 markedly attenuated this effect as observed in the MCT-AP group. No differences of RV hemodynamics between sham and sham-AP were observed. Therefore, the effects of Pyr-AP13 on RV hemodynamics were significantly different in sham and MCT animals, as confirmed by interaction analysis (Table 2).

Regarding the LV, although there was a tendency for the MCT group to present a smaller LV P_{max}, dP/dt_{max}, and dP/dt_{min} than sham animals, at this stage, no significant differences were observed, except an increase in τ. The effects of apelin on the LV were mostly observed on diastolic function as confirmed by the decrease of end-diastolic pressure (Table 2).

Morphometric analysis. RV chronic pressure overload secondary to PH resulted in RV hypertrophy as expressed by RV/LV + S and RV-to-BW ratios in MCT animals (Table 1). This RV mass gain was significantly prevented after Pyr-AP13 chronic treatment. Similarly, at the cellular level, the MCT group presented larger cardiomyocytes than sham animals. Chronic infusion of Pyr-AP13 significantly reduced cardiomyocyte hypertrophy in the MCT-AP group but did not alter the size of the cardiomyocyte in sham animals (Fig. 1*A*). Furthermore, collagen deposition was significantly increased in the MCT group, a change that was corrected by chronic administration of Pyr-AP13 as MCT-AP fibrosis values became comparable with those of the sham + AP group (Fig. 1*B*).

At the pulmonary level, PH significantly induced hypertrophy of the pulmonary vessels media in the MCT group, but this hypertrophy was not prevented by chronic administration of Pyr-AP13 (Fig. 2). In sham animals, apelin had no effect on mean vessel wall thickness.

Apelin and ANG-(1–7) plasmatic levels. Apelin-36 and ANG-(1–7) plasmatic levels were significantly increased in MCT animals (apelin, 0.77 ± 0.18 vs. 0.38 ± 0.13 ng/ml in sham, $P < 0.001$; and ANG-(1–7), 3.00 ± 1.7 vs. 1.15 ± 0.70 ng/ml in sham, $P < 0.01$). Pyr-AP13 chronic administration normalized apelin-36 and ANG-(1–7) plasmatic levels as observed in the MCT-AP group (0.44 ± 0.15 ng/ml, $P < 0.001$ vs. MCT; and ANG-(1–7), 0.99 ± 0.38 ng/ml in MCT-AP,

Table 2. *Chronic effects of apelin administration on hemodynamic parameters*

	Sham	Sham-AP	MCT	MCT-AP
		Right Ventricle		
P_{max}, mmHg	23.1±5.0	22.8±4.1	46.9±4.4*†	32.0±6.3*†‡§
EDP, mmHg	1.6±1.5	1.3±2.1	2.1±2.0	1.0±0.8
dP/dt_{max}, mmHg/s	899±193	867±149	1,632±259*†	1,087±311‡§
dP/dt_{min}, mmHg/s	−807±156	−759±115	−1,456±234*†	−984±278†‡§
t, ms	12.6±2.8	13.2±2.9	19.0±3.9*†	15.7±2.5‡§
		Left Ventricle		
P_{max}, mmHg	88.2±20.6	87.9±29.8	75.5±8.4	73.0±22.7
EDP, mmHg	3.4±1.6	2.1±2.1	2.8±2.0	1.4±0.7
dP/dt_{max}, mmHg/s	4,081±1,069	4,009±1,441	3,854±1,720	3,625±1,404
dP/dt_{min}, mmHg/s	−3,285±1,172	−3,393±1,337	−2,557±1,428	−2,667±749
τ, ms	13.7±0.9	12.1±1.5	17.4±3.4*†	15.2±1.8†‡

Values are means ± SD. P_{max}, peak systolic pressure; EDP, end-diastolic pressure; dP/dt_{max}, peak rate of ventricular pressure rise; dP/dt_{min}, peak rate of ventricular pressure decline; τ, time constant of isovolumetric relaxation. Comparisons were performed using two-way ANOVA followed by the Student-Newman-Keuls test to perform pairwise multiple comparisons. *$P < 0.05$ vs. Sham; †$P < 0.05$ vs. Sham-AP; ‡$P < 0.05$ vs. MCT; §significant interaction.

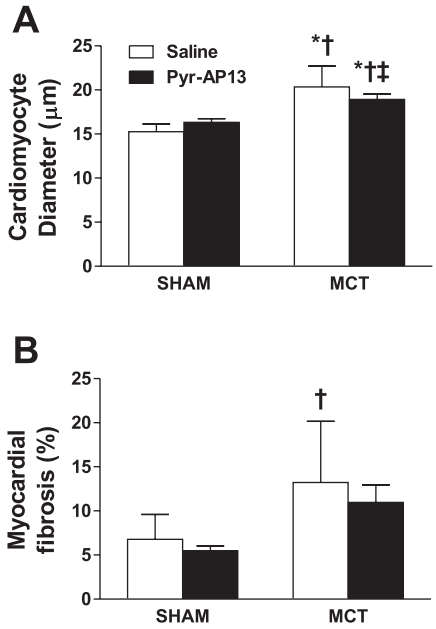

Fig. 1. Cardiomyocyte diameter (*A*) and myocardial fibrosis (percentage; *B*) in sham and moncrotaline (MCT) animals treated with saline or apelin [pyroglutamylated apelin-13 (Pyr-AP13)]. Data are means ± SD. *$P < 0.05$ vs. sham; †$P < 0.05$ vs. sham-AP; ‡$P < 0.05$ vs. MCT.

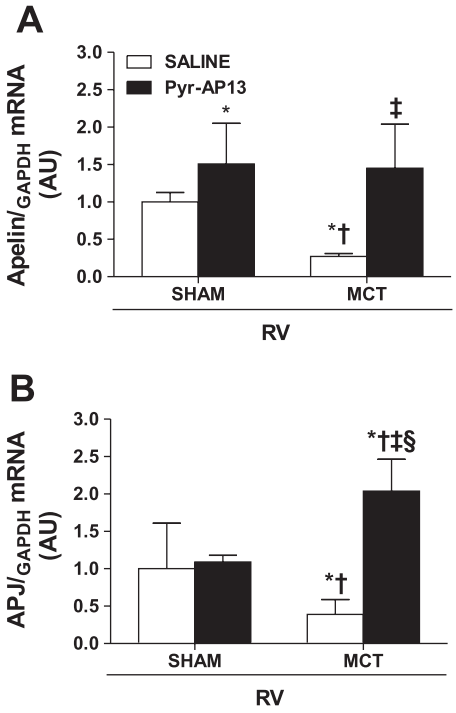

Fig. 3. Expression of apelin (*A*) and apelin receptor (APJ; *B*) in the right ventricle (RV). Results are normalized for GAPDH and presented in arbitrary units (AU). Data are means ± SD. *$P < 0.05$ vs. sham; †$P < 0.05$ vs. sham-AP; ‡$P < 0.05$ vs. MCT; §significant interaction.

$P < 0.05$ vs. MCT). In sham animals, no significant changes were observed after treatment with Pyr-AP13 (apelin, 0.36 ± 0.12 ng/ml, $P = 0.76$; and ANG-(1–7), 1.15 ± 0.78 ng/ml, $P = 0.46$).

Gene expression profile. RV expression of apelin and APJ was significantly decreased in the MCT animals, revealing a marked myocardial downregulation of this system in PH. Pyr-AP13 administration blunted this decrease. Interestingly, in sham-AP animals, exogenous apelin increased its endogenous myocardial expression (Fig. 3). Regarding the expression of genes typically involved in autocrine/paracrine activation during the progression of PH, ET-1, and ANG II, herein represented by its precursor angiotensinogen, showed an upregulation in the MCT group that was prevented by chronic administration of Pyr-AP13. Surprisingly, Pyr-AP13 treatment significantly increased the expression of Mas receptor either in sham or in MCT animals (Fig. 4). As expected from the myocardial hypertrophy data, the MCT group presented mRNA overexpression of BNP and Pyr-AP13 chronic administration remarkably reduced BNP mRNA levels in MCT-AP (Fig. 5). Interestingly, Pyr-AP13 chronic treatment decreased ET-1 in the nonoverloaded left ventricle (Fig. 6).

In pulmonary parenchyma of MCT-treated animals, apelin and APJ showed the same expression trends as in the RV; this is a decrease in the MCT group and an increase in the MCT-AP group. However, these differences did not reach statistical significance. In sham animals, the treatment with Pyr-AP13 did not change the expression of apelin. No differences between groups were observed in pulmonary ET-1 mRNA expression (Table 3).

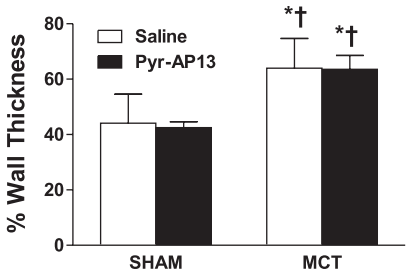

Fig. 2. Mean vessel wall thickness of peripheral pulmonary arteries (25–50 μm) in sham and MCT animals treated with saline or apelin (Pyr-AP13). Data are means ± SD. *$P < 0.05$ vs. sham; †$P < 0.05$ vs. sham-AP.

DISCUSSION

In the present study we documented the myocardial beneficial effects of apelin in MCT-induced pulmonary hypertension. The most relevant findings were that Pyr-AP13 treatment decreased PH, RV overload, hypertrophy, and myocardial neurohumoral activation, improved biventricular diastolic function, and normalized RV mRNA expression of apelin, APJ receptor, and BNP. Remarkably, exogenous Pyr-AP13 induced its endogenous myocardial expression both in healthy and in PH animals. Moreover, we demonstrated that apelin ameliorates PH progression not only by its well-known vasodilator effects but also by its direct modulation of myocardial neurohumoral activation. These findings extend and complement previously described cardiovascular effects of apelin in HF, suggesting an important cardiovascular role for the apelin-APJ system, namely as a powerful autocrine/paracrine myocardial modulator in PH.

Previous studies highlighted the importance of the apelin-APJ system in cardiac overload, reporting that chronic treatment with apelin resulted in increased dP/dt_{max}, dP/dt_{min}, and decreased LV end-diastolic pressure in a HF rat model induced by isoproterenol (21, 24, 35), whereas in healthy rats, it

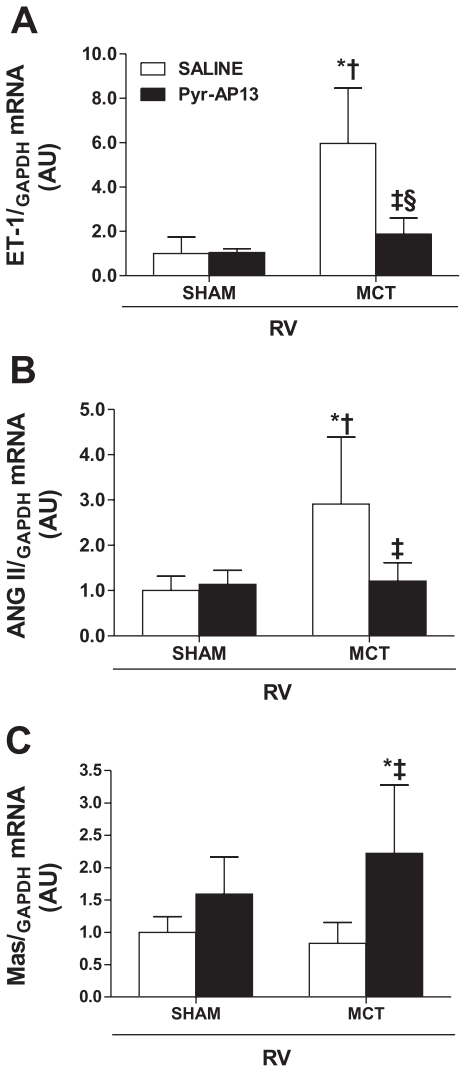

Fig. 4. Expression of endothelin-1 (ET-1; *A*), angiotensin II (ANG II; *B*), and Mas receptor (Mas; *C*) in the RV. Results are normalized for GAPDH and presented in arbitrary units (AU). Data are means ± SD. *$P < 0.05$ vs. sham; †$P < 0.05$ vs. sham-AP; ‡$P < 0.05$ vs. MCT; §significant interaction.

induced a significant increase on the velocity of circumferential shortening and cardiac output without signs of cellular hypertrophy (1). Furthermore, apelin knockout mice exhibited normal cardiac development in adulthood, which deteriorated with

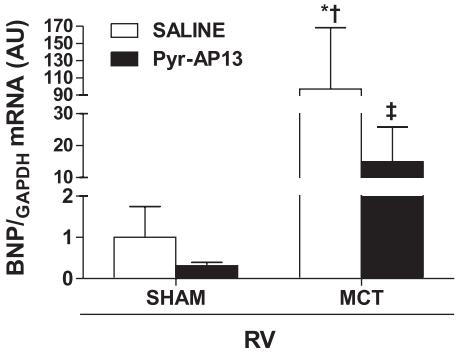

Fig. 5. Expression of type B natriuretic peptide (BNP) in the RV. Results are normalized for GAPDH and presented in arbitrary units (AU). Data are means ± SD. *$P < 0.05$ vs. sham; †$P < 0.05$ vs. sham-AP; ‡$P < 0.05$ vs. MCT.

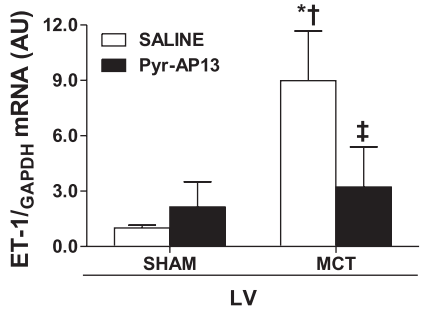

Fig. 6. Expression of ET-1 in the left ventricle (LV). Results are normalized for GAPDH and presented in arbitrary units (AU). Data are means ± SD. *$P < 0.05$ vs. sham; †$P < 0.05$ vs. sham-AP; ‡$P < 0.05$ vs. MCT.

aging or with chronic pressure overload. Interestingly, a continuous 2-wk infusion of apelin-13 in these elderly mice reversed the decreased contractility due to aging (24). Particularly, in a rat model of HF with hypoxia-induced PH, acute apelin administration in papillary muscles induced a positive inotropic effect that was greater in HF rats than in healthy rats, which was attributed to changes in increased intracellular calcium transients rather than changes in myofilament calcium responsiveness (10). Our results further strengthen and extend these previous observations by showing, for the first time, the beneficial cardiac effects of apelin under increased RV afterload conditions.

A unique characteristic of apelin, when compared with other known inotropic agents (2, 4), is the fact that it enhanced RV function while decreasing myocardial hypertrophy in PH animals. Even in sham animals it did not promote myocardial hypertrophy, reinforcing the previous finding of Ashley et al. (1) that demonstrated that apelin has the capacity to reduce LV preload and afterload and chronically increase cardiac output without inducing hypertrophy. Another interesting feature of this agent relates to its effect on the extracellular matrix: apelin normalized myocardial fibrosis in the MCT-AP group to values similar to those of the sham group, which is in consonance with a previous study that demonstrated an upregulation of collagen IV mRNA and other sets of genes involved in myocardial fibrosis in an apelin knockout mice (24).

Contrasting with the important vasodilator effects previously described in vasculature (8, 20, 39), Pyr-AP13 failed to prevent pulmonary structural damages induced by MCT administration, namely, to attenuate the hypertrophy of the media in small peripheral pulmonary arteries. However, prognosis and survival in PH has been related predominantly with the integrity of RV function rather than with the degree of pulmonary vasculature injury (29).

In the present study, MCT animals showed a significant reduction of apelin and APJ receptor mRNA expression in the RV. Our findings are supported by previous observations in

Table 3. *Pulmonary gene expression profile*

	Sham	Sham-AP	MCT	MCT-AP
Apelin/$_{GAPDH}$ mRNA, AU	1.00±0.74	1.08±0.85	0.46±0.34	1.63±1.68
APJ/$_{GAPDH}$ mRNA, AU	1.00±0.86	0.96±0.69	0.20±0.12	0.82±0.53
ET-1/$_{GAPDH}$ mRNA, AU	1.00±1.20	1.12±1.06	0.19±0.11	0.47±0.50

Values are means ± SD. ET-1, endothelin-1; APJ, apelin receptor. Results are normalized for GAPDH and presented in arbitrary units (AU).

cultured neonatal rat ventricular myocytes subjected to mechanical stretch (35) and also in in vivo models of chronic RV (10) and LV (42) pressure overload. Remarkably, chronic exogenous administration of Pyr-AP13 induced an increase in the endogenous myocardial expression of apelin and Mas receptor both in healthy and in PH animals although the exact mechanisms remain to be clarified. In our study, we can speculate that the increased production of plasmatic apelin-36 in the MCT-treated rats might represent a compensatory response to overcome the downregulation of the apelinergic system in the injured RV. Previous studies have reported contradictory findings regarding the circulating levels of apelin in patients with HF [elevated (7), decreased (9, 14, 17), or unchanged (27)], suggesting its potential dependence on the underlying cardiac pathology. Currently, it is accepted that plasmatic levels of apelin are increased in the early stages of heart failure but decreased in later stages (7, 8, 14), a fact that has been suggested to promote a transient compensatory response.

So far, ACE2 is the only breakdown enzyme for apelin, and therefore its activity, herein accessed by the formation of ANG-(1–7) from ANG II, is increased likely as a compensatory response to the increased plasmatic levels of apelin of the MCT group. Furthermore, considering the well-known vasodilator properties of apelin (20, 39) and ANG-(1–7) (13), increased plasmatic levels of these agents could locally modify the excessive production of vasoconstrictors, such as ANG II and ET-1, and decrease RV pressure overload induced by hypertrophied media of pulmonary vessels. Despite apelin strong vasodilator effects, the mechanical afterload reduction is not the only mechanism behind PH amelioration. As herein demonstrated, apelin markedly attenuated the PH-induced neurohumoral activation by preventing ANG II and ET-1 cardiac overexpression and consequently their deleterious effects. This results from a direct myocardial modulation of apelin since it prevented ET-1 overexpression in the nonoverloaded LV. Moreover, Zisman et al. (43) demonstrated that ANG-(1–7) is produced in the intact human heart, is counterregulatory of ANG II, and is decreased when ANG II formation is suppressed by an ACE inhibitor, suggesting that a major pathway for the formation of ANG-(1–7) was directly dependent on the availability of ANG II as a substrate. Interestingly, although ANG-(1–7) effects are to a large extent mediated by the Mas receptor (33, 37), the mRNA expression of this receptor in the RV does not seem to be related with the plasma levels of ANG-(1–7).

Increased circulating levels and myocardial upregulation of ET-1 have been widely reported in PH (28), with the extent of such increase being directly proportional to the severity of the disease (15). Elevated endogenous ET-1 production, acting through endothelin receptor ET_A, causes pulmonary vasoconstriction that is normally masked by endogenous NO production and contributes to the progression of cardiopulmonary alterations in MCT-induced PH (15, 28). In the present study, ET-1 mRNA levels were increased in the overloaded RV and also in the nonoverloaded LV of MCT animals and normalized in the MCT-AP group. In fact, our laboratory has previously demonstrated that MCT-treated rats with long-standing PH presents autocrine/paracrine system activation of the LV in the absence of a direct hemodynamic stress, myocardial hypertrophy, or fibrosis. In the present study, we confirmed this aspect and additionally showed that Pyr-AP13 treatment of the MCT rats directly modulates myocardial neurohumoral environment of the overloaded and nonoverloaded myocardium.

Regarding ANG II, several authors have reported ANG II and ACE upregulation in the MCT-induced PH rat model (26, 40). In APJ-deficient mice, Ishida and collaborators (20) demonstrated the role of apelin-APJ system against the vasopressor action of ANG II. Accordingly, the present study showed a normalization of the increased ANG II overexpression in the MCT-AP group. This strongly suggests that apelin is a new neurohumoral modulator, acting as a counterregulatory peptide for ET-1 and ANG II vasoconstrictor properties. Moreover, the significant reduction of BNP mRNA expression in MCT-AP animals certainly confirms the effectiveness of Pyr-AP13 chronic treatment in decreasing RV pressure overload and ameliorating the myocardial injuries induced by PH.

Although further work is required to delineate the pathways by which exogenous apelin modulates PH-induced neurohumoral activation as well as its myocardial production, we have demonstrated that RV and pulmonary deleterious effects of MCT-induced PH are accompanied by abnormalities of the apelin-APJ system. The proposed mechanism suggests that in the course of PH the decreased expression of the apelinergic system in the myocardium triggers a compensatory increase of plasmatic apelin, raising ACE2 activity and consequently ANG-(1–7) formation. Both vasodilator species might help to reduce PH-induced RV pressure overload. Chronic Pyr-AP13 administration started early in the course of PH was able to *1*) upregulate apelin myocardial expression and prevent a concomitant increase of its plasmatic levels, *2*) prevent apelin and APJ mRNA downregulation and ensure a normal vasodilator effect of apelin-APJ system, and *3*) blunt ET-1 and ANG II upregulation showing its modulator role in myocardial neurohumoral activation. Altogether these effects show that Pyr-AP13 treatment decreases RV overload and hypertrophy, improves biventricular diastolic function, and so ameliorates PH. These findings suggest a possible role of the apelin-APJ system in the pathophysiology of PH, as well as its use as a potential therapeutic target in this disease.

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