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Manuel João Neves Ferreira Pinto

Survivin Role in Pulmonary Arterial Hypertension

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Dedico este trabalho aos meus exemplos.

À minha mãe Helena e à minha madrinha Ana Maria, que me ensinam todos os dias o que é a transcendência e o amor incondicional.

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Abstract

In the present work we characterized the morphological and hemodynamic progression of monocrotaline (MCT)-induced pulmonary arterial hypertension (PAH) and evaluated survivin expression in the right ventricle (RV) throughout the natural history of the disease. We tested the effects of the survivin-antagonist terameprocol in pulmonary artery smooth muscle cells (PASMC) proliferation and apoptosis. Adult male Wistar rats received a subcutaneous injection of MCT (60 mg/Kg) or equal volume of vehicle. On days 1, 3, 7, 14 and 21 after injection (n=7-12 per group per time-point), right ventricular pressures were measured, heart and lungs were weighted and RV and lung samples were collected for histological analysis. Survivin and smac/DIABLO expression in the RV was determined by immunohistochemistry. In a different protocol, a primary culture of PASMC isolated from sham and MCT-treated rats was established and the effects of terameprocol in cell proliferation and apoptosis were evaluated by BrdU and TUNEL assays, respectively. Our results demonstrate that survivin upregulation and smac/DIABLO downregulation in the RV precede hemodynamic manifestations of PAH and pair RV hypertrophy, strongly suggesting a role in cardiac remodeling. Terameprocol halted cell proliferation and induced apoptosis of PASMC from both sham and pulmonary hypertensive rats. Our results suggest that targeting survivin in PAH could have dual beneficial effects, by reversing pulmonary vascular remodeling and cardiac hypertrophy.

Keywords

Pulmonary Arterial Hypertension; Apoptosis; Survivin; Smac/DIABLO; Terameprocol.

Introduction

Pulmonary arterial hypertension (PAH) is a disorder of the pulmonary vasculature, associated with increased pulmonary vascular resistance and pulmonary arterial pressure(25, 37). By acquiring a hyperproliferative and apoptosis-resistant phenotype(12, 23, 24, 52, 56), pulmonary artery smooth muscle cells (PASMC) play a pivotal role in the vascular remodeling distinctive of PAH. The observation that PAH shares several pathophysiologic mechanisms with neoplastic disorders impelled the creation of the cancer paradigm of PAH(62, 77). In fact, it is possible to identify in PAH almost all of the 6 hallmarks of cancer postulated by Hanahan and Weinberg in 2000(34). Only tissue invasion and metastization have not been described in PAH(4, 66).

Further supporting the notion that PASMC in PAH express a neoplastic-like phenotype, McMurtry et al(53) observed that survivin was expressed in PASMC of patients and rats with PAH, but was absent in pulmonary arteries of control subjects. In the same study, gene therapy targeting survivin induced apoptosis and inhibited proliferation of PASMC, reverting MCT-induced PAH in rats and improving survival. Survivin is the smallest member of the "inhibitor of apoptosis" (IAP) protein family and regulates both mitosis and apoptosis(7). Diffusely expressed during embryonic and fetal development(3, 45, 76), survivin is virtually undetected in most fully differentiated adult tissues, with few exceptions(2, 11, 27, 32). However, survivin is upregulated in the majority of human cancers(9) and its expression has been correlated with decreased overall survival, increased rate of recurrence, resistance to therapy and reduced apoptotic index(10).

Released to the cytoplasm in response to apoptotic stimuli, survivin interacts with another member of the IAP family, XIAP (X-linked IAP)(21). This interaction protects XIAP from ubiquitin-mediated degradation and enhances its anti-caspase activity. It is believed that the increased release of mitochondrial survivin to the cytosol is essential for the apoptosis resistant phenotype of neoplastic cells(20). Smac/DIABLO is another

mitochondrial protein that is released to the cytosol when the mitochondrial apoptosis pathway is activated(17, 22, 81). Smac/DIABLO interacts with survivin and other IAP proteins(74) and prevents their inhibition of caspases, thus promoting apoptosis(22). Contrariwise, survivin upregulation in neoplastic cells also seems to sequester smac/DIABLO in the mitochondria, preventing its release to the cytosol and consequent pro-apoptotic action(16, 72).

Pharmacological modulation of survivin in PAH would probably be more promptly translated into clinical care than gene therapy, if demonstrated to be similarly effective. In this context, terameprocol (tetra-O-methyl nordihydroguaiaretic acid, M4N, EM1421), a derivate of the nordihydroguaiaretic acid (NDGA) isolated from the plant *Larrea tridentata(19, 39)*, suppresses survivin gene expression(18, 61) by binding to the transcription factor Sp1. *In vitro*, terameprocol, induced apoptosis and growth arrest of various human cancer cell lines(18, 28, 35, 46, 49, 57, 61). *In vivo* studies with mouse models of human xenograft solid tumors also attested the anti-tumoral activity of this compound, which was not accompanied by relevant systemic toxicity(28, 35, 46, 49, 61). Clinical trials are already being conducted to assess the potential of terameprocol in several types of neoplasias(33, 44, 71, 84) and prevention of sexually transmited viruses(43) (www.clinicaltrials.gov database, accessed in 10/02/2012).

Survivin relationship with PAH may not be limited to its upregulation in PASMC. Recent studies point out a crucial role of survivin in cardiac remodeling in the setting of heart failure(1, 47, 48, 80). Additionally, cardiomyocyte apoptosis is recognized as a major feature of right heart failure(14, 15). However, right ventricular (RV) survivin expression in the setting of PAH remains uninvestigated. In this work, we investigate cardiac expression of survivin and smac/DIABLO throughout the hemodynamic and morphometric progression of monocrotaline (MCT)-induced PAH. We also characterized the effects of terameprocol in PASMC isolated from sham and pulmonary hypertensive rats.

Materials and Methods

Chemicals and Drugs

Collagenase type 1 was from Worthington Biochemical Corp. (Lakewood, NJ). Trypsin and DMEM were from PAN Biotech (Aidenbach, Germany). HBSS and penicillin-streptomycin-amphotericin B were from Invitrogen (Madrid, Spain). Papain, DTT, bovine serum albumin, fetal bovine serum (FBS), sodium pyruvate, nonessential aminoacids, dimethylsulfoxide (DMSO) and terameprocol were from Sigma (Barcelona, Spain). Terameprocol was dissolved in DMSO. The maximal concentration of DMSO used in our experiments was 0.1%, which demonstrated no cytotoxic effect in our cell culture model (data not shown). Rabbit polyclonal anti-survivin and anti-smac/DIABLO antibodies, Streptavidin Protein and 3'-diaminobenzidine were from Abcam (Cambridge, United Kingdom).

Experimental Design

Animal experiments were performed according to the Portuguese law for animal welfare and conform to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 85-23, Revised 1996). Adult male Wistar rats (Charles River Laboratories; Barcelona, Spain) weighing 180–200 g were housed in groups of 5 rats /cage in a controlled environment under a 12:12-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 (60 mg/kg body wt, Sigma; Barcelona, Spain) (MCT groups) or an equal volume of vehicle (2 ml/kg body wt) (SHAM groups).

Hemodynamic evaluation and tissue sampling

On days 1, 3, 7, 14 or 21 after MCT/vehicle injection, 7-12 rats per group per time-point 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. A pressure–volume (PV) catheter was implanted in the RV (PVR-1045, Millar Instruments, Houston, TX). After complete instrumentation, the animal preparation was allowed to stabilize for 15 min. Hemodynamic recordings were made under basal conditions with respiration suspended at end-expiration. Data was continuously acquired (MPVS 300, Millar Instruments), digitally recorded at 1000 Hz (ML880 PowerLab 16/30, Millar TM Instruments), and analyzed (PVAN 3.5, Millar Instruments). RV pressure was measured at end-diastole and peak systole (P_{max}). Peak rates of RV pressure rise (dP/dt_{max}) and pressure decline (dP/dt_{min}) were measured as well.

Morphometric analysis

After complete hemodynamic assessment, animals were euthanized by exsanguination under anesthesia. The heart, lungs and right gastrocnemius muscle were excised and weighed. The right tibia was also excised and its length was measured with a millimetric ruler. The RV free wall was dissected from the left ventricle (LV) + septum (S), under binocular magnification (x3.5), and weighed separately. Heart, lungs, RV, and LV + S weights were normalized to body weight (BW) and gastrocnemius weight was normalized to tibial length. RV weight was also normalized to that of LV+S.

RV and right lung samples were immersion fixed in 4% paraformaldehyde 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 observed at microscope, photographed with a digital camera and measured with a digital image analyzer (cell^B life science basic imaging software, Olympus). All the measurements were made directly at x400 magnification (10), only in muscle fibers whose cross section included a nuclear profile.

RV samples were divided into five sections and the area of fifty cardiomyocyte per sample was measured and averaged.

On the pulmonary specimens, external diameter and medial wall thickness in muscular arteries (12-18 arteries/lung) were analyzed at x400 magnification. Orthogonal intercepts were used to generate eight random measurements of external diameter (distance between the external lamina) and sixteen random measurements of medial thickness (distance between the internal and external lamina). For each artery, medial hypertrophy was expressed as follows: %wall thickness = [(medial thickness x 2)/(external diameter)] x 100.

Immunohistochemistry

To determine survivin and smac/DIABLO expression in the RV, immunohistochemistry was performed on 4µm sections of paraformaldehyde fixed paraffin wax embedded ventricular tissue. After deparaffinization, slides were immersed in 10 mM Sodium Citrate Buffer pH 6.0 and subjected to heat induced antigen retrieval in microwave for 30 min. Rabbit polyclonal anti-survivin and anti-smac/DIABLO antibodies were used as primary antibody at 1:500 and 1:200 dilution, respectively and incubated overnight at 4°C. Streptavidin Protein was used as detection system. Sections were developed with 3'-diaminobenzidine and counterstained with Mayer's hematoxilin before mounting. Negative controls were obtained by omitting the primary antibody. The stained sections were observed using a light microscope at x400 magnification. Survivin and smac/DIABLO expression was qualitatively determined as positive (cytoplasmic staining) or negative.

Isolation and primary culture of PASMC

21 days after MCT/vehicle injection, rats were euthanized with an intraperitoneal injection of penthobarbital (120 mg/kg) (n=3 for each group). The left upper lung lobe

was removed and placed in a calcium enriched Hank's Buffered Salt Solution (HBSS) (mM: KCl 5,4; NaCl 137; KH2PO4 0,44; NaHCO3 4,2; NaH2PO4 0,25; D-glucose 1; phenol red 0,2; CaCl2 2 and MgCl2 1). First order intrapulmonary artery was dissected free of connective tissue under a stereomicroscope (Leica EZ4). After extraction from the lung, the adventitia was removed, the vessel was opened longitudinally and endothelium was removed by gently rubbing the inner surface with forceps tips.

In order to release smooth muscle cells, the artery, mainly consisting of medial layer, was submitted to an enzymatic dissociation process with papain and collagenase type 1, in a calcium-poor HBSS solution (25 μM CaCl2 and 1mg/mL BSA). Next, a mechanical dissociation process was performed with a fire polished, silicone coated glass pipette, in order to release the cells. The cell-free tissue was removed and the solution was centrifuged (5 minutes, 250g) in order to pellet the cells. Finally, cells were seeded in DMEM culture medium (supplemented with 1% penicillin-streptomycin-amphotericin B, 1% sodium pyruvate and 1% nonessential aminoacids) containing 10% FBS, in a 24-well culture plate (500 μL/well) and placed in an incubator (37ºC, 5%CO_2). Cell passage was performed when ≈80% confluence was achieved. Cells between passages 2 and 4 were used for all experiments. Smooth muscle origin was confirmed by typical morphology (fusiform shape with hills and valleys) and detection of smooth muscle α-actin expression by immunocytochemistry (data not shown). Mycoplasma detection tests were not performed.

For BrdU and TUNEL assays, cells were seeded in 24-well plates at a density of 1,5 x 10^4 cells /well. After 72 hours, medium was removed and cells were incubated for 24h in medium without FBS, containing different concentrations of terameprocol, or vehicle (DMSO). Each condition was tested in triplicate and in cells from three animals per group.

Cell proliferation assays

We evaluated the effect of terameprocol in cell proliferation using the 5'-bromodeoxyuridine (BrdU) immunoassay (Roche Diagnostics). BrdU is a thymidine analogue and its incorporation in the DNA is a measure of cell proliferation. BrdU incorporation immunoassay was conducted following the 24h incubation with terameprocol/vehicle. All protocol was followed according to manufacturer's instructions. Results are given as mean ± standard error (SE) and are expressed as percentage of control (medium with vehicle).

Apoptosis assay

For apoptosis evaluation, we used the TUNEL (TdT-mediated dUTP Nick End Labelling) In Situ Cell Death Detection kit (Roche Diagnostics), according to the manufacturer's instructions. After the 24h incubation of cells with terameprocol/vehicle, cells were stained with TUNEL and DAPI. The percentage of apototic cells was calculated by dividing the number of cells stained with TUNEL (apoptotic cells) by the total number of nuclei stained with DAPI (Roche Diagnostics), in at least 10 different fields at 200x magnification. Results are given as mean ± SE.

Statistical analysis

Statistical analysis was performed using Sigma Stat 3.5 software.

Group data are presented as means ± SE 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 Holm-Sidak test was selected to perform pairwise multiple comparisons. Statistical significance was set at $P < 0.05$.

Results

Morphometric analysis

The morphometric progression of MCT-induced PAH is resumed in Table 1.

Regarding to body weight, on day 21, the MCT group exhibited a significant decrease compared to the SHAM group. In the SHAM groups, body weight significantly increased between contiguous time-points. Contrariwise, the body weight of MCT treated animals failed to significantly increase between the time-points: D1 to D3, D3 to D7 and D14 to D21.

In the MCT groups, lung weight increased predominantly between D14 and D21. In fact, this parameter was significantly higher in D21 compared to the other time-points evaluated. In the SHAM groups, no differences were found in the lung weight parameter.

In the SHAM group, we observed a reduction of the relation HT/BW on D14 and D21, but no variation in MCT group. MCT-treated animals developed RV hypertrophy, as expressed by the indexes RV/(LV+S) and RV/BW, both significantly increased on D21. There were no differences in the (LV+S)/BW parameter between SHAM and MCT groups.

As expressed in Figure 1, MCT induced an increase in the RV cardiomyocyte cross-sectional area, which was significant since day 7 after injection and progressively increased until day 21. Pulmonary arterial wall thickness was significantly higher in MCT-treated rats 14 and 21 days after injection (Figure 2). The peak of pulmonary arterial wall thickness was on day 14 and partially regressed on day 21, but still being higher than in the SHAM group.

RV Hemodynamics

As presented on Table 2, hemodynamic manifestations of PAH started on day 14 after MCT injection. At this time-point, RV peak systolic pressure was significantly augmented in the MCT group, and further increased until day 21. On days 14 and 21 after injection,

dP/dt_{max} and dP/dt_{min} were significantly higher (absolute values) in the MCT groups comparing to the respective SHAM groups, but there was no progression from day 14 to day 21 in the MCT groups.

RV survivin and smac/DIABLO expression

RV survivin and smac/DIABLO expression are shown in Figures 3 and 4. Survivin was virtually unexpressed in the RV of sham rats. In the MCT group, survivin expression was sparsely present on day 3 after injection and became evident on day 7, further increasing throughout days 14 and 21. Inversely, smac/DIABLO expression progressively decreased with time in the MCT groups and was relatively constant in the SHAM groups.

PASMC proliferation

The effect of terameprocol in PASMC proliferation is plotted in Figure 5. 24h treatment with terameprocol (10, 20 and 50 μM) significantly inhibited proliferation of cells from sham and pulmonary hypertensive rats, in a dose-dependent manner, while the lower doses (0.1 and 1 μM) did not differ from control. The pattern of proliferation did not differ between sham and pulmonary hypertensive cells, except for the terameprocol dose of 20 μM. This concentration inhibited less markedly the proliferation of pulmonary hypertensive cells, comparing with the SHAM group.

PASMC Apoptotis

The pro-apoptotic activity of terameprocol is displayed on Figure 6. While the lower doses had no effect on apoptosis, the higher doses tested (20 and 50 μM) induced apoptosis in cells from both sham and pulmonary hypertensive rats, in a dose-dependent manner. At 20 μM, terameprocol induced a significantly higher percentage of apoptotis in the SHAM group compared to the MCT.

Discussion

The rat model of MCT-induced PAH is one of the most widely used. Although not all the typical findings of the human disease are mimicked by this model (like plexiform lesions), MCT induces pulmonary artery medial layer remodeling and RV hypertrophy(75), which are the focus of our study. In rats, the severity of the lesions induced by MCT depends on aspects such as the MCT dose, the administration route and the animal age at the time of treatment(73, 75).

Almost all the research conducted in animal models has been focusing on severe stages, like D25-35 after MCT injection, probably because most patients with PAH first present with advanced-stage disease. To our knowledge, since Todd's study in 1985(75), research on the early features of PAH has been scarce. In the present study, we investigated the natural progression of MCT-induced PAH and identified the timing of appearance of its hemodynamic and morphological features. It is our conviction that more attention should be paid to the early events of the disease in order to identify and modulate new pathophysiologic pathways that could lead to a cure.

An elegant study by Levkau et al(48) unraveled several key functions of survivin in the heart. Cardiac survivin-deficient mice presented in the neonatal period with a reduced total cardiomyocyte number and marked cardiomyocyte polyploidy. This phenotype ultimately led to progressive heart failure and death. Additionally, overexpression of survivin in cultured neonatal cardiomyocytes induced cell division and apoptosis-resistance. Finally, this study found only residual survivin expression in normal adult human hearts, but a remarkable overexpressed in the myocardium of patients with end-stage heart failure. Curiously, after hemodynamic support with LVAD survivin cardiomyocyte expression was significantly reversed, raising the possibility that cardiac survivin expression is load-dependent. In the present study, RV expression of survivin increased progressively throughout the development of MCT-induced PAH. Survivin overexpression started as early as D7 after MCT injection, the time-point that

corresponded to the first evidence of cardiomyocyte hypertrophy. These findings strongly suggest that survivin might be involved in the myocardial remodeling process. The pattern of progressive increase in survivin expression during the development of the disease supports the Levkau's hypothesis. Interestingly, survivin overexpression preceded hemodynamic manifestations of the disease, which only started at D14. McMurtry's study also revealed that lung survivin expression preceded hemodynamic disease(53). However this is understandable given that pulmonary arterial remodeling is the primary pathogenic mechanism of MCT-induced PAH. We propose that pulmonary vascular remodeling phenomena may be signalized to the RV, possibly by a neurohumoral mediator, which induces survivin overexpression and cardiomyocyte hypertrophy. Cardiac hypertrophy allows the thin-walled RV to withstand the pressure overload, while survivin overexpression inhibits cardiomyocyte loss by apoptosis and, possibly, induces mitosis of cardiomyocytes or cardiac progenitor cells. In fact, a study with a rat model of spontaneously hypertensive rats found that myocardial expression of survivin in ageing and heart failure is inversely correlated with cardiac apoptosis and associated with a more favorable cardiac remodeling (1). Endothelin-1, angiotensin-II, catecholamines and platelet-derived growth factor (PDGF) are possible neurohumoral candidates responsible for survivin upregulation in the RV (14). A positive feedback relationship between PDGF and survivin was demonstrated to induce smooth muscle cell proliferation and apoptosis resistance in arterial injury models(13, 78). A cardioprotective role of survivin has also been suggested in the setting of ischemia/reperfusion injury. In fact, insulin(69), δ-opioid receptor activation(83) and ischemic preconditioning(41) upregulate survivin expression in cardiomyocytes, inducing an apoptosis-resistant state, associated with increased myocardial viability in the peri-infarct area(67).

Contrasting with survivin, smac/DIABLO expression progressively decreases throughout the development of PAH. The pro-apoptotic properties of smac/DIABLO

arise from its interaction with survivin and other IAP proteins(74), preventing their anti-caspase action(22). Inversely, excessive survivin also sequesters smac/DIABLO in the mitochondria, inhibiting its pro-apoptotic function(16, 72). Therefore, we hypothesize that the balance between the antiapoptotic survivin and the proapoptotic smac/DIABLO could be determinant for the cardiomyocyte fate in response to apoptotic stimuli. Several studies have also demonstrated a reciprocal expression of survivin and Smac/DIABLO in different cancers(30, 51, 54, 58, 59).

In the past two decades, the introduction of agents that target the excessive vasoconstriction and SMC proliferation [prostacyclin analogues(29, 60, 70), endothelin-1 receptor antagonists(63, 65) and phosphodiesterase-5 inhibitors(31, 68)] improved quality of life and prolonged survival of patients with PAH. Nevertheless, they do not revert nor halt the progression of the disease. Therefore, there is no cure for PAH yet and the available therapies are scarce(26, 38). A limited effect on vascular remodeling might explain the unsatisfactory outcome of vasodilator therapies. In fact, there has been a shift in the interest of the scientific community to seek antiproliferative therapies rather than new vasodilators(36, 40, 64). The hyperproliferative and apoptosis-resistant phenotype of PASMC in PAH constitutes an attractive therapeutic target. In 2006, McMurtry et al reversed MCT-induced PAH with anti-survivin gene therapy (53). In the last decade, the arsenal of anti-survivin drugs has been expanding, and several agents are already in clinical trials(5, 6, 8, 42, 50, 55, 79, 82). In this context, pharmacological modulation of survivin in PAH would probably be more promptly translated into clinical care than gene therapy, if similarly effective. The anti-survivin agent terameprocol was shown to inhibit proliferation and stimulate apoptosis in transformed cancer cell lines. Additionally, some animal and clinical trials did not demonstrate relevant systemic toxicity(28, 35, 46, 49, 61). In this work we characterized the antiproliferative and pro-apoptotic effects of terameprocol in PASMC isolated from sham and pulmonary

hypertensive rats. These cell culture results support further research on this topic, namely with direct administration of terameprocol to pulmonary hypertensive rats.

In conclusion, survivin seems to be an attractive therapeutic target in PAH. The anti-survivin agent terameprocol may be a candidate, acting at the pulmonary level by inhibiting proliferation and inducing apoptosis of PASMC, and at the RV level, by counteracting myocardial hypertrophy.

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Figure captions

Figure 1: Right ventricular cardiomyocyte hypertrophy, expressed as cardiomyocyte cross-sectional area (μm^2). RV, right ventricle; SHAM, sham group; MCT, monocrotaline group. Data are means ± SE. *P < 0.05 vs. SHAM of the same day; ^{a}P < 0.05 vs. D14 of the same treatment group, ^{b}P < 0.05 vs. D7 of the same treatment group; ^{c}P < 0.05 vs. D3 of the same treatment group; ^{d}P < 0.05 vs. D1 of the same treatment group.

Figure 2: Pulmonary arterial hypertrophy. A to J: Histological appearance of small pulmonary arteries, stained with hematoxylin and eosin. A-E: SHAM group; F-J: monocrotaline (MCT) group. K: medial layer thickness expressed as percentage of wall thickness. Data are given as means ± SE. *P < 0.05 vs. SHAM of the same day; ^{a}P < 0.05 vs. D14 of the same treatment group, ^{b}P < 0.05 vs. D7of the same treatment group; ^{c}P < 0.05 vs. D3 of the same treatment group; ^{d}P < 0.05 vs. D1 of the same treatment group.

Figure 3: Survivin expression in the right ventricle, evaluated by immunohistochemistry during the progression of pulmonary hypertension. SHAM, sham group; MCT, monocrotaline group. Time-points evaluated were days 1, 3 7, 14 and 21 after injection.

Figure 4: Smac/DIABLO expression in the right ventricle, evaluated by immunohistochemistry during the progression of pulmonary hypertension. SHAM, sham group; MCT, monocrotaline group. Time-points evaluated were days 1, 3 7, 14 and 21 after MCT or vehicle injection.

Figure 5: Effect of terameprocol in pulmonary artery smooth muscle cell proliferation. Cells were isolated from sham and pulmonary hypertensive animals, and proliferation evaluated by the BrdU incorporation assay. TMP, terameprocol; SHAM,

sham group; MCT, monocrotaline group. Data are expressed as percentage of the absorbance of control (TMP 0 μM) from the same group, and given as means ± SE. *P < 0.05 vs. control of the same group; #P < 0.05 vs. SHAM of the same TMP concentration; aP < 0.05 vs. 20 μM of the same group; bP < 0.05 vs. 10 μM of the same group; cP < 0.05 vs. 1 μM of the same group; dP < 0.05 vs. 0.1 μM of the same group.

Figure 6: Effect of terameprocol in pulmonary artery smooth muscle cell apoptosis. Cells were isolated from sham and pulmonary hypertensive animals and apoptosis was evaluated by the TUNEL assay. TMP, terameprocol; SHAM, sham group; MCT, monocrotaline group. Data are expressed as percentage of apoptotic cells and given as means ± SE. *P < 0.05 vs. control of the same group (TMP 0 μM); #P < 0.05 vs. SHAM of the same TMP concentration; aP < 0.05 vs. 20 μM of the same group; bP < 0.05 vs. 10 μM of the same group; cP < 0.05 vs. 1 μM of the same group; dP < 0.05 vs. 0.1 μM of the same group.

Tables

Table 1: Morphometric progression of monocrotaline-induced pulmonary arterial hypertension.

	D1		D3		D7		D14		D21	
	SHAM	MCT	SHAM	MCT	SHAM	MCT	SHAM	MCT	SHAM	MCT
Body weight (g)	194,1 ± 5,1	195,4 ± 5,9	211,0 ± 4,7 d	198,6 ± 1,7	230,4 ± 8,2 c,d	214,6 ± 3,2	262,2 ± 9,5 b,c,d	253,7 ± 6,0 b,c,d	290,6 ± 4,5 a,b,c,d	260,8 ± 4,9 *,b,c,d
HT/BW (g/Kg)	3,271 ± 0,028	3,243 ± 0,091	3,242 ± 0,047	3,189 ± 0,059	3,031 ± 0,079	3,129 ± 0,061	2,862 ± 0,037 c,d	3,025 ± 0,051	2,794 ± 0,069 c,d	3,282 ± 0,113 *
RV/ (LV+S) (g/g)	0,264 ± 0,014	0,265 ± 0,021	0,268 ± 0,013	0,260 ± 0,005	0,282 ± 0,017	0,332 ± 0,021	0,287 ± 0,018	0,320 ± 0,011	0,302 ± 0,012	0,467 ± 0,049 *,a,b,c,d
RV/BW (g/Kg)	0,578 ± 0,025	0,591 ± 0,040	0,578 ± 0,023	0,590 ± 0,016	0,583 ± 0,038	0,665 ± 0,036	0,560 ± 0,032	0,608 ± 0,021	0,584 ± 0,013	0,911 ± 0,095 *,a,b,c,d
(LV+S)/ BW (g/Kg)	2,197 ± 0,037	2,240 ± 0,037	2,165 ± 0,030	2,274 ± 0,061	2,070 ± 0,058	2,011 ± 0,047 c,d	1,956 ± 0,037 c,d	1,900 ± 0,026 c,d	1,957 ± 0,060 c,d	1,956 ± 0,042 c,d
L/BW (g/Kg)	5,731 ± 0,260	5,592 ± 0,117	5,426 ± 0,202	5,311 ± 0,140	5,436 ± 0,307	5,896 ± 0,400	4,661 ± 0,173	5,639 ± 0,220 *	4,782 ± 0,323	7,241 ± 0,464 *,a,b,c,d
G/tib (g/cm)	0,341 ± 0,008	0,337 ± 0,023	0,348 ± 0,008	0,345 ± 0,014	0,382 ± 0,012	0,374 ± 0,004	0,423 ± 0,020	0,416 ± 0,013	0,440 ± 0,016	0,430 ± 0,008

Data are means ± SE. SHAM, sham group; MCT, monocrotaline group; RV, right ventricle; LV + S, left ventricle plus septum; HT, heart; BW, body weight; L, lung; tib, tibia, G, gastrocnemius. *P < 0.05 vs. SHAM of the same day; [a]P < 0.05 vs. D14 of the same treatment group, [b]P < 0.05 vs. D7of the same treatment group; [c]P < 0.05 vs. D3 of the same treatment group; [d]P < 0.05 vs. D1 of the same treatment group.

Table 2: Right ventricular hemodynamic progression of monocrotaline-induced pulmonary arterial hypertension.

	D1		D3		D7		D14		D21	
	SHAM	MCT	SHAM	MCT	SHAM	MCT	SHAM	MCT	SHAM	MCT
Heart rate (bpm)	378 ± 23	413 ± 14	410 ± 18	387 ± 20	349 ± 22	357 ± 10	358 ± 35	385 ± 30	340 ± 14	376 ± 23
Peak Systolic Pressure (mmHg)	28,0 ± 1,9	28,3 ± 2,1	27,2 ± 0,8	28,2 ± 1,6	27,3 ± 2,2	31,5 ± 1,5	23,7 ± 1,0	31,9 ± 1,3 *	26,3 ± 1,2	38,9 ± 2,7 *,a,b,c,d
End-diastolic Pressure (mmHg)	3,9 ± 0,5	2,7 ± 0,3	3,2 ± 0,3	2,7 ± 0,3	3,9 ± 0,3	4,2 ± 1,0	3,9 ± 0,2	3,4 ± 0,7	3,6 ± 0,4	3,5 ± 0,3
dPdt max (mmHg/sec)	1671 ± 152	1848 ± 184	1901 ± 90	2102 ± 217	1685 ± 155	1906 ± 83	1405 ± 101	2233 ± 190 *	1563 ± 60	2257 ± 65 *
dPdt min (mmHg/sec)	-1544 ± 355	-1421 ± 189	-1629 ± 109	-1702 ± 230	-1624 ± 204	-1441 ± 142	-1020 ± 144	-1830 ± 172 *	-1228 ± 113	-2263 ± 93 *,b,d

Data are means ± SE. SHAM, sham group; MCT, monocrotaline group. *P < 0.05 vs. SHAM of the same day; [a]P < 0.05 vs. D14 of the same treatment group, [b]P < 0.05 vs. D7of the same treatment group; [c]P < 0.05 vs. D3 of the same treatment group; [d]P < 0.05 vs. D1 of the same treatment group.

Figure 1: Right ventricular cardiomyocyte hypertrophy.

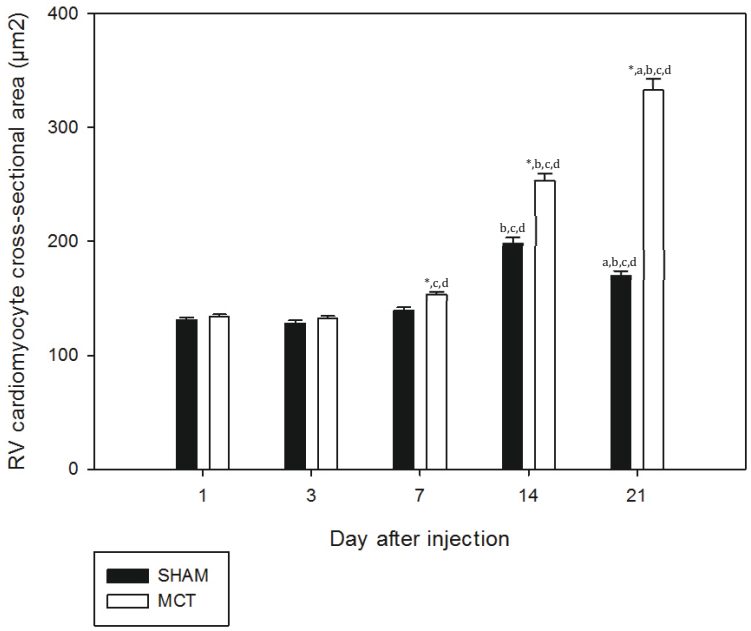


Figure 2: Pulmonary arterial hypertrophy.

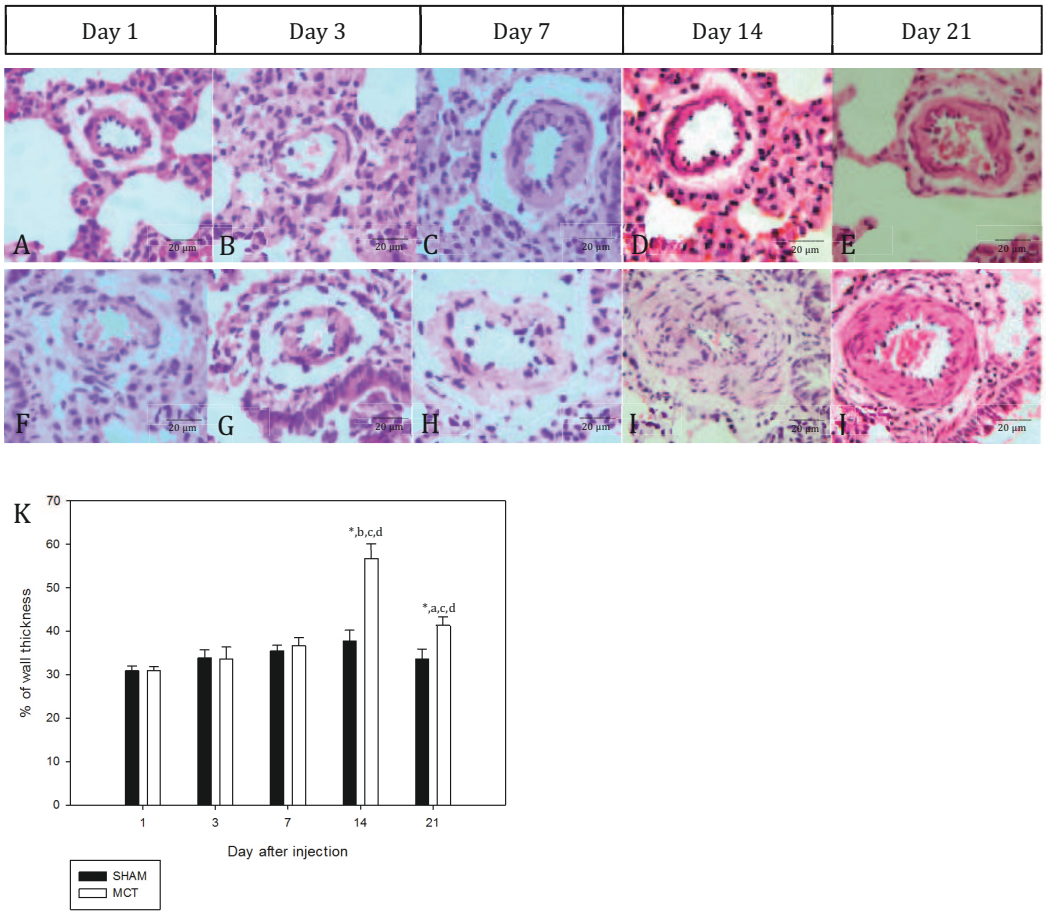


Figure 3: Survivin expression in the right ventricle.

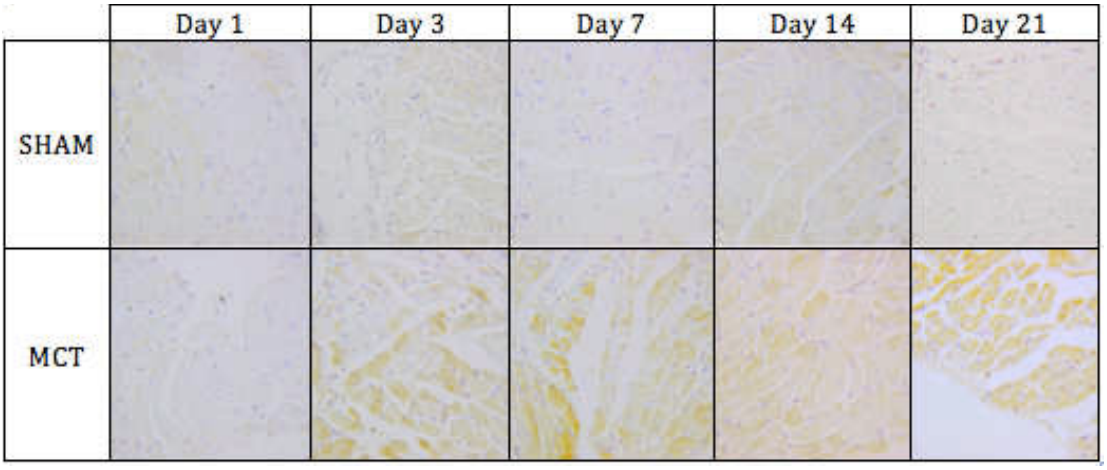


Figure 4: Smac/DIABLO expression in the right ventricle.

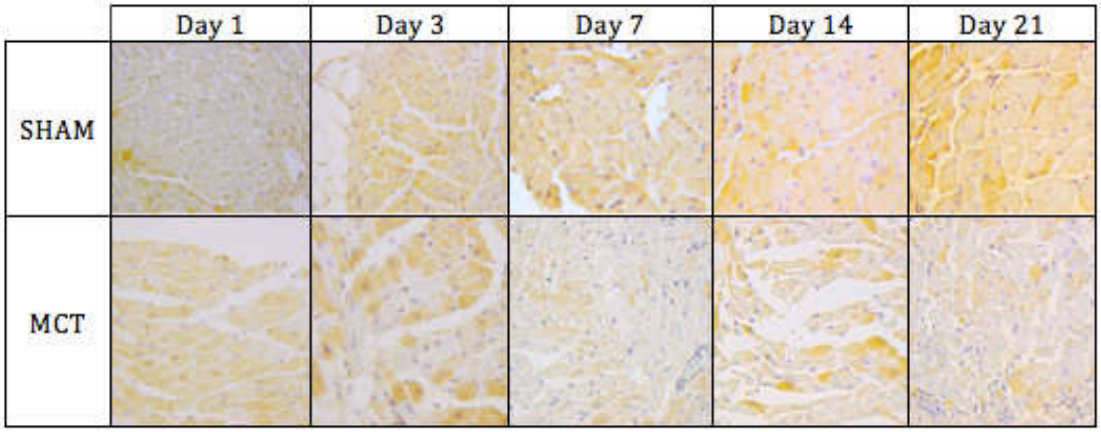


Figure 5: Effect of terameprocol in pulmonary artery smooth muscle cell proliferation.

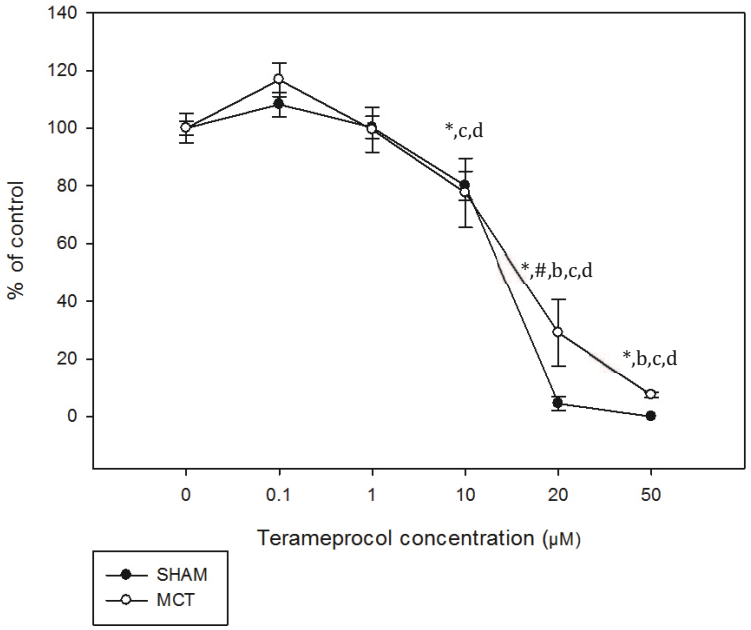
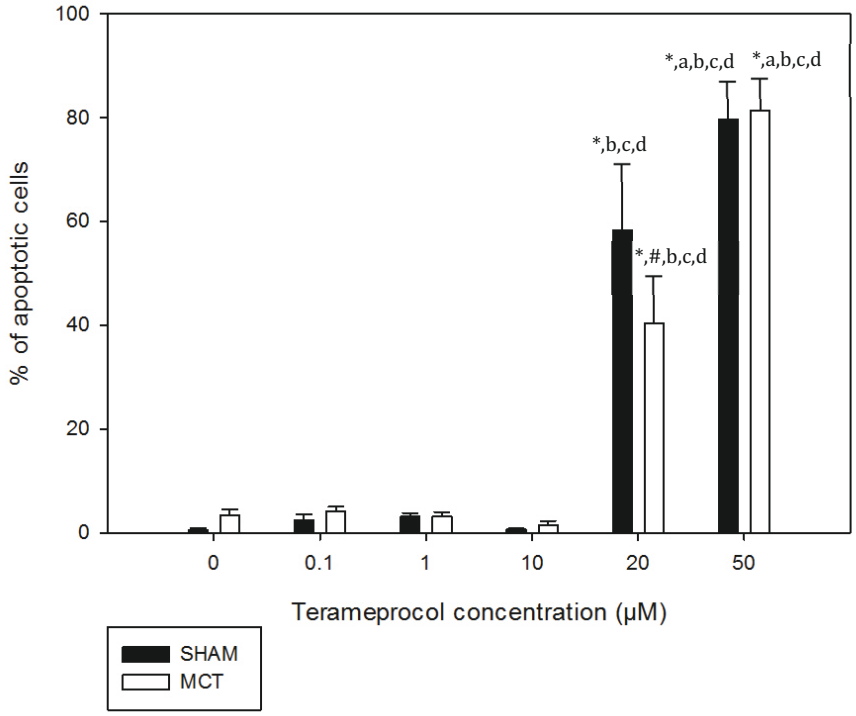


Figure 6: Effect of terameprocol in pulmonary artery smooth muscle cell apoptosis.



Annex

American Journal of Physiology - Heart And Circulatory Physiology

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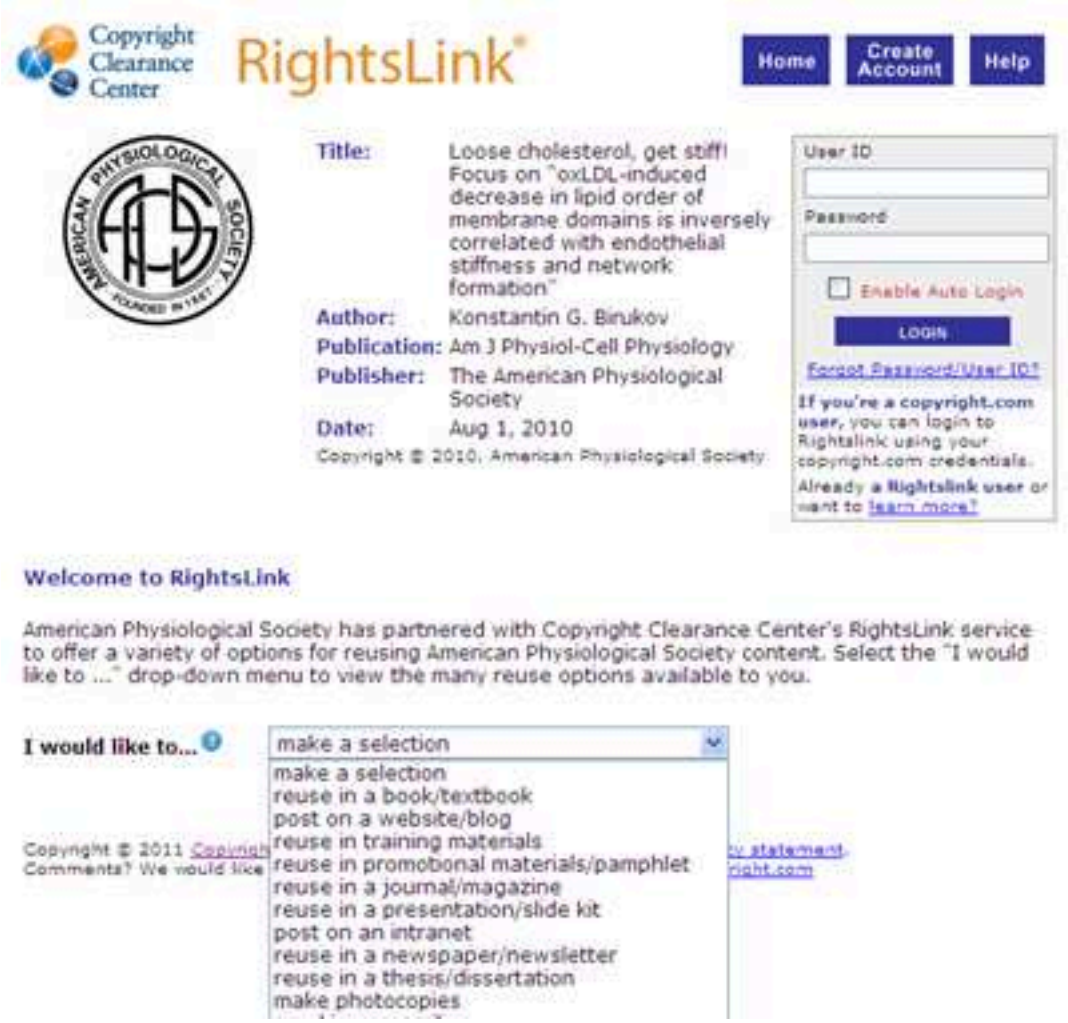

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1. Villalobos AR, Parmelee JT, Renfro JL. Choline uptake across the ventricular membrane of neonate rat choroid plexus. *Am J Physiol Cell Physiol* 276: C1288-C1296, 1999.

2. Pollock DM. Endothelin receptor subtypes and tissue distribution. In: *Endothelin Molecular Biology, Physiology, and Pathology*, edited by Highsmith RF. Totowa, NJ: Humana, 1998.

3. Scarafia LE, Winter A, Swinney DC. Quantitative expression analysis of the cellular specificity of HECT-domain ubiquitin E3 ligases. *Physiol Genomics* (April 26, 2001). doi:10.1152/physiolgenomics.00075.2001.

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