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Thyrotropin-releasing hormone overexpression induces structural changes of the left ventricle in the normal rat heart

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Schuman ML, Peres Diaz LS, Landa MS, Toblli JE, Cao G, Alvarez AL, Finkielman S, Pirola CJ, García SI. Thyrotropin-releasing hormone overexpression induces structural changes of the left ventricle in the normal rat heart. *Am J Physiol Heart Circ Physiol* 307: H1667–H1674, 2014. First published October 3, 2014; doi:10.1152/ajpheart.00494.2014.—Thyrotropin-releasing hormone (TRH) hyperactivity has been observed in the left ventricle of spontaneously hypertensive rats. Its long-term inhibition suppresses the development of hypertrophy, specifically preventing fibrosis. The presence of diverse systemic abnormalities in spontaneously hypertensive rat hearts has raised the question of whether specific TRH overexpression might be capable of inducing structural changes in favor of the hypertrophic phenotype in normal rat hearts. We produced TRH overexpression in normal rats by injecting into their left ventricular wall a plasmid driving expression of the preproTRH gene (PCMV-TRH). TRH content and expression of preproTRH, collagen type III, brain natriuretic peptide, β-myosin heavy chain, Bax-to-Bcl-2 ratio, and caspase-3 were measured. The overexpression maneuver was a success, as we found a significant increase in both tripeptide and preproTRH mRNA levels in the PCMV-TRH group compared with the control group. Immunohistochemical staining against TRH showed markedly positive brown signals only in the PCMV-TRH group. TRH overexpression induced a significant increase in fibrosis, evident in the increase of collagen type III expression accompanied by a significant increase in extracellular matrix expansion. We found a significant increase in brain natriuretic peptide and β-myosin heavy chain expression (recognized markers of hypertrophy). Moreover, TRH overexpression induced a slight but significant increase in myocyte diameter, indicating the onset of cell hypertrophy. We confirmed the data "in vitro" using primary cardiac cell cultures (fibroblasts and myocytes). In conclusion, these results show that a specific TRH increase in the left ventricle induced structural changes in the normal heart, thus making the cardiac TRH system a promising therapeutic target.

heart; rat; thyrotropin-releasing hormone; fibrosis

THYROTROPIN-RELEASING HORMONE (TRH), a small neuropeptide (p-Glu-His-Pro-NH_2) initially identified in the hypothalamus, is amply distributed in the central nervous system (17) and in other extra neural tissues (4). TRH also acts on the cardiovascular system of rodents, increasing blood pressure, heart rate, and contractility after its intracerebroventricular or intravenous administration (15). The hypertensive effect of TRH seems to be independent from the TRH-thyroid stimulating hormone (TSH)-triiodothyronine (T_3) system. In fact, in almost all of our protocols, we measured thyroid hormones [T_3 and thyroxine (T_4)] and TSH, confirming these results (6). Indeed, other laboratories have demonstrated the existence of different pools of TRH neurons around the central nervous system, indicating that there are many TRH-induced effects that do not involve TSH stimuli.

Previous research has demonstrated *1*) that the TRH system is present in the rat heart (3), *2*) the existence of a positive inotropic effect of TRH on the guinea pig myocardium (9), *3*) that TRH induces cardiac contractility of isolated rat hearts (24), *4*) the enhancement of ventricular contractility by TRH in ventricular myocytes (26), and *5*) elevation of the TRH system in the rat heart after myocardial infarction (11). This body of evidence suggests that a local TRH system may participate in the cardiac damage/remodeling process.

The spontaneously hypertensive rat (SHR) model has been extensively used for studying human essential hypertension. This model presents a gradual progression of myocardial pathological hypertrophy, fibrosis, left ventricular (LV) dysfunction, and heart failure, with the upregulation of genes encoding extracellular matrix (ECM) components (2).

We have recently reported that adult SHR shows LV TRH hyperactivity and that long-term inhibition of LV TRH by naked small interfering (si)RNA prevents LV fibrosis and cardiomyocyte enlargement and attenuates the expression of brain natriuretic peptide (BNP), demonstrating successful inhibition of cardiac hypertrophy in a rat model by cardiac-targeted ablation of TRH production (23).

Data from other laboratories have indicated that even though fibroblasts are the primary source of preproTRH in the heart, this precursor has also been observed in myocytes (11, 12). The specific type 1 TRH receptor has also been found in the heart (26) and belongs to the G protein family (25), which includes the angiotensin II type 1 receptor and the endothelin type A receptor, both inductors of hypertrophy and fibroblast proliferation. In addition, infiltration or expansion of fibroblasts in cardiac tissue can lead to an increase in the deposition of collagens and other ECM components.

As speculated by Jankowski (10), TRH released from fibroblasts may contribute to the cross-talk between cardiac fibroblasts and myocytes during the development of hypertrophy. Indeed, multiple kinase pathways that stimulate these processes, such as PKC, Ca^{2+}/calmodulin-dependent kinase, MAPK, etc., are activated by TRH (10).

Although further investigations would be needed to elucidate the transduction signal pathways activated by TRH in

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heart tissue, these results raise a number of questions, including a very important one: is the single upregulation of TRH expression and activity a sufficient step predisposing LV fibrosis as well as other structural changes in the normal heart?

Thus, we speculated that specific LV TRH overexpression could be able to induce fibrosis and maybe hypertrophic features in normal hearts.

MATERIALS AND METHODS

Ethics statement. The Institutional Animal Care and Use Committee approved the animal experimentation protocols following their ethical guidelines. The protocol was approved by CICUAL UE-IDIM (IDIM-CONICET, Buenos Aires, Argentina). All protocols were performed under anesthesia, and all efforts were made to minimize suffering.

All reagents were from Sigma (St. Louis, MO) unless otherwise indicated.

Animals. Eight-week-old male Wistar rats (10 rats/group, Charles River Laboratories) were used. Animals were housed in a room with controlled temperature (23 ± 1°C) under a 12:12-h light-dark schedule.

"In vivo" TRH overexpression. The pcDNA3 plasmid (5.4 kb) was used (Invitrogen). Rat TRH cDNA was cloned downstream of the strong cytomegalovirus promoter (PCMV-TRH). The control group received empty pcDNA3 (PCMV-Con). Rats were assigned in a random blind fashion to one of two groups, PCMV-TRH or PCMV-Con (n = 10 rats/group), and anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg). Previously, we performed a dose curve (25, 50, and 100 μg plasmid/injection), and we used 100 μg, which showed the maximal induction. In our hands, PCMV transfection induced TRH expression during >7 days; indeed, injections were conducted weekly during the experiment (4 wk) to maintain the induction. All injections were performed under echography. The chests of the rats were shaved and cleaned. Gel was liberally applied to the exterior body surface before placement of the probe. The probe was aligned with the needle, and special care was taken to visualize the region of the myocardial wall. Injection was performed in three different sites (from the base to apex) to efficiently surround the entire LV as previously described (23, 16).

All experiments were also performed blindly with respect to the treatment. At the end of the protocol, rats were decapitated, and hearts were removed.

Isolation and culture of neonate rat ventricular myocytes and fibroblasts. We used neonatal cell culture to confirm "in vitro" TRH overexpression results, as this has been vastly reported has several advantages for studies examining cellular response, among them, cultured neonatal cells can be more easily transfected than adult cells. Wistar neonatal rat ventricular myocytes and fibroblasts were isolated and prepared as previously described (22). Briefly, myocytes were dispersed from ventricles of 1- to 3-day-old Wistar rats by digestion with collagenase type II and trypsin at 37°C (GIBCO). The cell suspension was separated on a discontinuous Percoll gradient to obtain myocardial cell cultures with >95% myocytes. Myocytes were preplated in 10-cm culture dishes in DMEM containing 100 μg/ml streptomycin and 100 U/ml penicillin supplemented with 10% FBS for 1–2 h to remove nonmyocyte cells. Unattached myocytes were removed, and equal numbers of cells were plated on 35-mm collagen type I-coated plates. To inhibit noncardiac myocyte growth, 100 μmol/l bromodeoxyuridine was also added.

After the Percoll gradient, the layer of fibroblasts was plated in 10-cm culture dishes in DMEM containing 100 μg/ml streptomycin and 100 U/ml penicillin supplemented with 10% FBS.

In all experiments, myocytes or fibroblasts were deprived of serum and incubated for another 24 h before treatment.

Cell transfection. Transfection of cardiac cells was performed using Lipofectamine 2000 (Invitrogen) based on the manufacturer's recommendations. For optimization of the procedure, the ratios of Lipofectamine to PMCV plasmid DNA concentration (in μl/μg) were selected to get the best result (5/1). Expression of TRH was evaluated using quantitative real-time RT-PCR.

TRH content. For peptide extraction, cardiac tissue samples were collected in acetic acid (2 mol/l) and HCl (0.1 mol/l). All samples were boiled for 20 min and centrifuged at 5,000 *g*, and the supernatant was lyophilized. The residue was dissolved in the appropriate buffer for the measurement of TRH-like immunoreactivity by RIA or the mobile phase for the identification of authentic TRH by HPLC. This procedure and the RIA for TRH have been previously reported in detail (6).

Quantitative real-time RT-PCR. Quantification of preproTRH, TRH receptor 1, prohormone convertase 1 (PC1), collagen type III, BNP, atrial natriuretic peptide (ANP), and caspase-3 mRNA expression was performed using a real-time RT-PCR technique normalized by β-actin housekeeping gene expression. Briefly, for cDNA synthesis, 2 μg of total RNA (from tissue or cells) and 1 μg of random

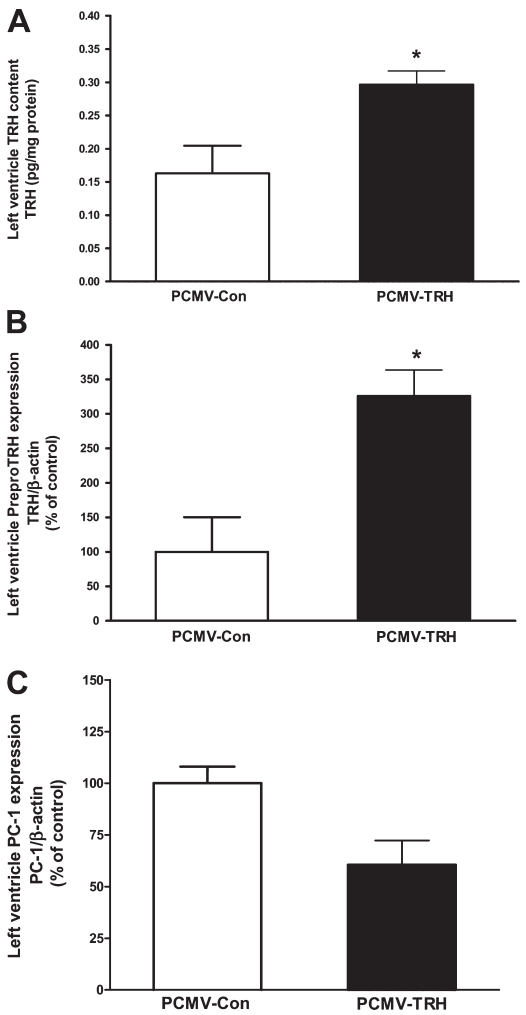

Fig. 1. Left ventricular (LV) thyrotropin-releasing hormone (TRH) overexpression in Wistar rats. *A–C*: LV TRH content (in pg/mg protein) measured by RIA (*A*) and TRH precursor (preproTRH; *B*) and prohormone convertase 1 (PC1; *C*) gene expression determined by real-time PCR normalized by β-actin expression in Wistar rats injected with the PCDNA3 plasmid encoding the preproTRH precursor gene (PCMV-TRH) or the empty PCDNA3 plasmid used as a control (PCMV-Con). n = 6 rats/group. *P < 0.04 between groups.

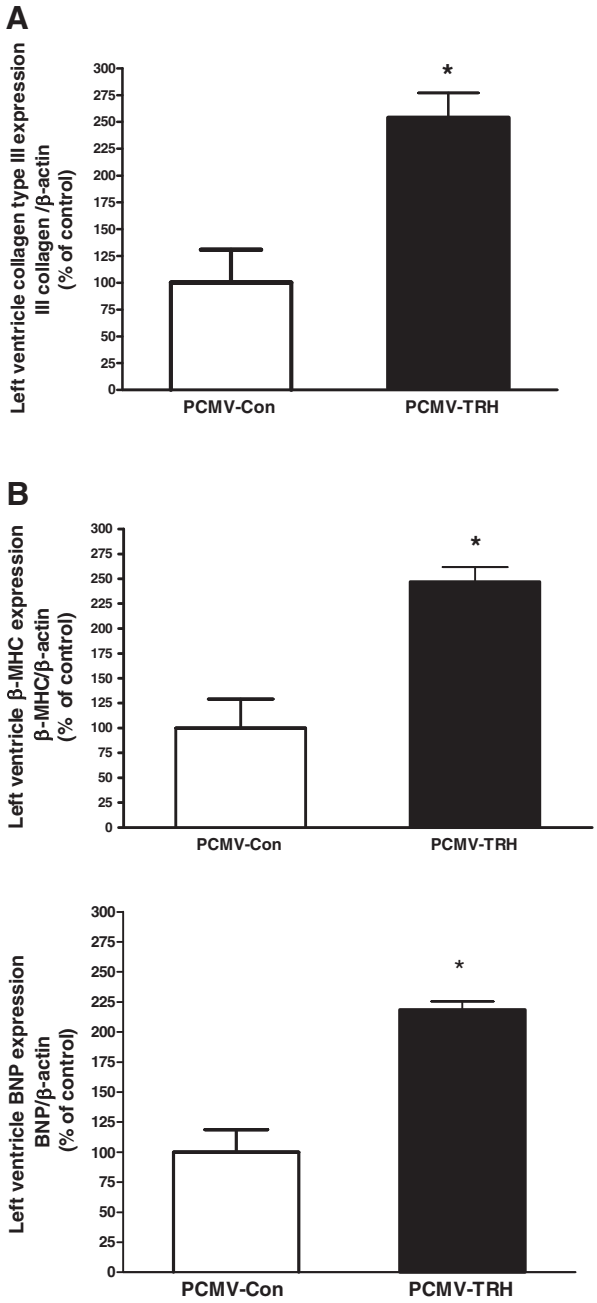

Fig. 2. Effect of LV TRH overexpression on fibrotic and hypertrophic markers. *A* and *B*: collagen type III (*A*), brain natriuretic peptide (BNP; *B*), and β-myosin heavy chain (β-MHC; *B*) mRNA expression were determined by real-time PCR and normalized by β-actin expression. Results are expressed as percentages of the control group; $n = 6$ rats/group. *$P < 0.03$ compared with the PCMV-Con group.

hexamer primers (Promega) were heated followed by incubation at 4°C. After RT reaction (Promega), real-time PCR was performed using a Bio-Rad iQ iCycler Detection System (Bio-Rad Laboratories) with SYBR green fluorophore. Reactions (in duplicate) were carried out in a total volume of 20 μl. A three-step protocol (95°C for 30 s; 60, 64, or 66°C annealing for 30 s; and 72°C extension for 40 s) was repeated for 40 cycles. Rat primers (Invitrogen) for rat preproTRH, rat BNP, rat collagen type III, and rat β-actin were as previously described (23). Additional primers were designed as follows: rat Bax, forward 5′-CTGCAGAGGATGATTGCTGA-3′ and reverse 5′-GGGCACTTTAGTGCACAGG-3′; rat Bcl-2, forward 5′-GATAACGGAGGCTGGGATG-3′ and reverse 5′-CTCACTTGTGGCCCAGGTAT-3′; and rat caspase-3, forward 5′-CAAGTCGATGGACTCTGGAA-3′ and reverse 5′-TGACATTCCAGTGCTTTTATGG-3′.

Melt curve analysis was performed after every run to ensure a single amplified product for every reaction. The authenticity of the amplicons generated was confirmed by their size on a 2% agarose gel. In accordance with the literature, we did not find any differences between experimental groups in the housekeeping gene β-actin.

Pathology examination. Hearts were perfused with saline solution through the abdominal aorta until they were free of blood. Tissue was fixed in phosphate-buffered 10% formaldehyde (pH 7.2) and embedded in paraffin. Sections (3 μm) were cut and stained with hematoxylin and eosin, Masson's trichrome, Azan-Mallory, and Sirius red. All observations in light microscopy were performed using a Nikon E400 light microscope (Nikon Instrument Group).

Immunohistochemical TRH and caspase-3 evaluation. Immunolabeling was carried out using a modified avidin-biotin-peroxidase technique (Vectastain ABC kit, Universal Elite, Vector Laboratories). After deparaffinization and rehydration, sections were washed in PBS for 5 min. Quenching of endogenous peroxidase activity was achieved by incubating sections for 30 min in 1% hydrogen peroxide in methanol. After being washed in PBS (pH 7.2) for 20 min, sections were incubated with blocking serum for a further 20 min. Thereafter, sections were rinsed in PBS and incubated with biotinylated universal antibody for 30 min. After a final wash in PBS, sections were incubated for 40 min with Vectastain Elite ABC reagent (Vector Laboratories) and exposed for 5 min to 0.1% diaminobenzidine (Polyscience) and 0.2% hydrogen peroxide in 50 mmol/l Tris buffer (pH 8). For the purpose of evaluating TRH content, as in the RIA technique, the specific anti-rat TRH polyclonal antibody was used at a dilution of 1:100.

Apoptosis was assessed using a rabbit polyclonal anti-active caspase-3 antibody (Chemicon) at a dilution of 1:50. In every heart, positive immunostaining for caspase-3 was expressed as number of cells with positive nuclear staining per area.

Morphological and quantitative analyses. All tissue samples were evaluated independently by two investigators without prior knowledge of the group to which the rats belonged. All measurements were carried out using an image analyzer (Image-Pro Plus version 4.5 for Windows, Media Cybernetics). Histomorphometric evaluation of the heart was carried out according to the following schedule: ECM expansion and numeric density of myocytes and coronary capillaries

Table 1. *Effect of LV TRH overexpression on morphometric variables*

Morphometric Variables	PCMV-TRH Group	PCMV-Con Group	*P* Value
LV wall			
Cardiomyocyte diameter, μm	26.3 ± 0.7	23.8 ± 0.6	<0.04
Cardiomyocyte density, number of cardiomyocytes/area	11.5 ± 0.3	12.8 ± 0.4	<0.05
Number of capillaries/area	25.6 ± 0.5	28.1 ± 0.6	<0.02
Myocyte-to-capillary ratio	0.44 ± 0.03	0.45 ± 0.03	NS
IVS			
Cardiomyocyte diameter, μm	26.4 ± 0.7	23.6 ± 0.6	<0.02
Cardiomyocyte density, number of cardiomyocytes/area	11.4 ± 0.3	12.8 ± 0.3	<0.03
Number of capillaries/area	25.4 ± 0.6	27.9 ± 0.5	<0.02
Myocyte-to-capillary ratio	0.44 ± 0.06	0.45 ± 0.06	NS

Values are means ± SE. LV, left ventricular; TRH, thyrotropin-releasing hormone; PCMV-TRH group, group injected with the PCDNA3 plasmid encoding the preproTHR precursor gene; PCMV-Con group, group injected with the empty PCDNA3 plasmid (control group); IVS = interventricular septum; NS, not significant.

(vessels < 8 μm) within the confines of each of the 20 random views at ×400 magnification.

Biochemical and pathology experiments. At the end of the treatment, rats were weighed, and the snout-tail long index was measured before rats were killed by decapitation. Blood samples were collected with sodium EDTA, and thyroid hormone levels were measured using an enzyme immunometric assay (Assay Designs). Hearts were rapidly removed and weighed. In some animals, cardiac tissues were separated (atria, LV, right ventricle, and septum) for TRH determination by RIA and for the quantification of mRNA expression; others were used for pathology experiments.

Systolic arterial blood pressure measurement. Animals were acclimated in a quiet room for 30 min before the measurement of systolic arterial blood pressure (SABP) by a tail-cuff method weekly during the experiment.

Statistics. Values are expressed as means ± SE. All statistical analyses were performed using absolute values and processed using Statistics (version 6.0, Software). The assumption test to determine Gaussian distribution was performed by the Kolmogorov-Smirnov method. For parameters with Gaussian distribution, comparisons between groups were carried out using one-way ANOVA followed by Bonferroni's test, Kruskal-Wallis test (nonparametric ANOVA), and Dunn's multiple comparison test when appropriate. In the case of two groups, comparison was performed using a *t*-test or Mann-Whitney test when appropriate. *P* values of <0.05 were considered significant.

RESULTS

Following the described protocol, we found significant increases in both TRH content (nearly 2-fold) and preproTRH mRNA expression (>3-fold) in the LV of animals injected with PCMV-TRH with respect to control rats injected with the empty plasmid (PCMV-Con, $P < 0.04$), indicating the effectiveness of the LV TRH overexpression design (Fig. 1, *A* and *B*). We also tested the rest of the heart chambers and found no significant changes in the right ventricle and atria, although a small increase was also seen in the septum compared with the PCMV-Con group, which did not reach statistical significance. In contrast, although there was a tendency, no significant differences were observed in PC1 gene expression between the groups (Fig. 1*C*), even though Perello et al. (20) have reported that PC1 is a specific convertase involved in proTRH processing as a regulator step in TRH activity in many tissues.

To confirm the specificity of TRH overexpression, prepro-TRH gene expression was evaluated in other heart tissues of

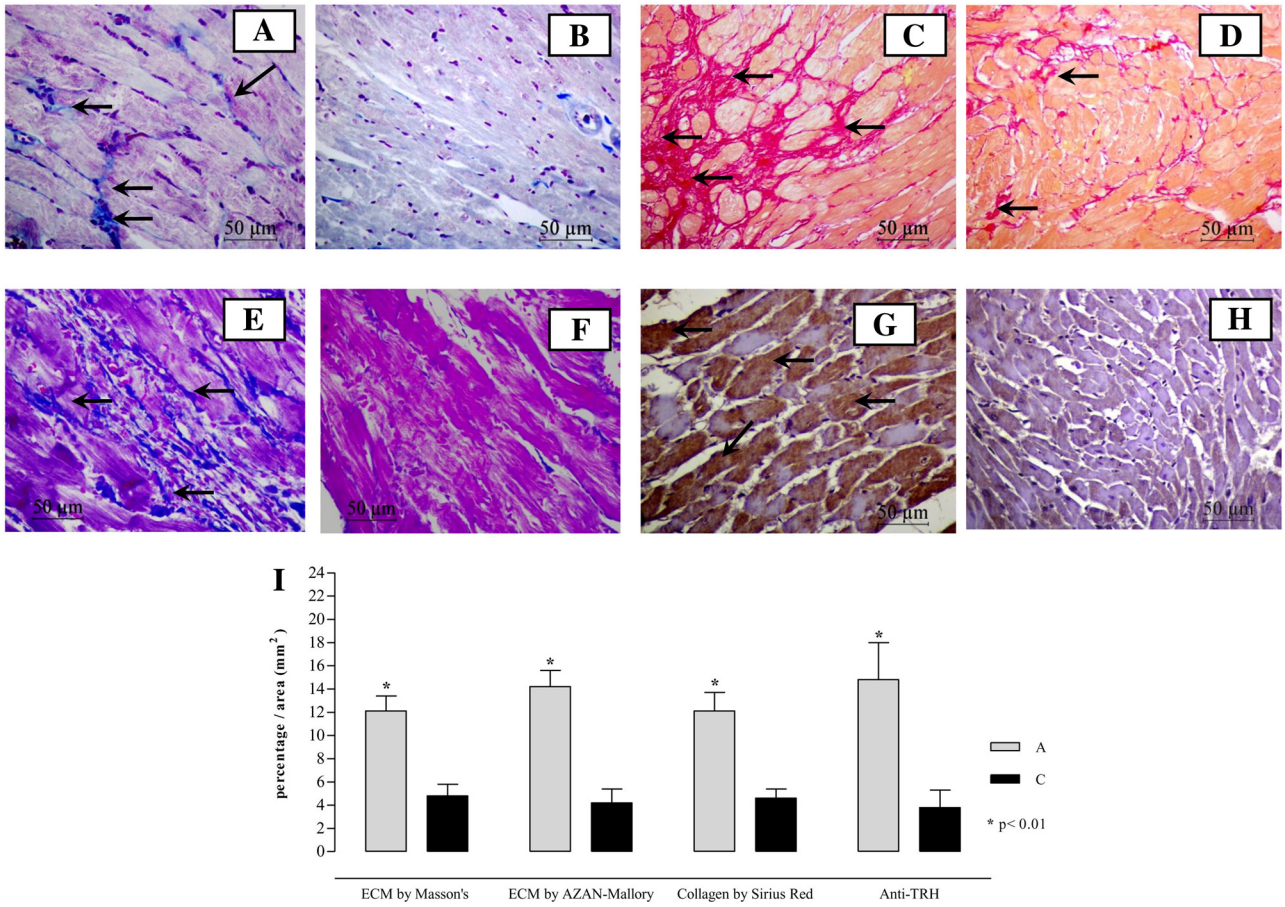

Fig. 3. Effect of LV TRH overexpression on extracellular matrix (ECM) expansion, collagen, and cardiomyocyte morphometry. Magnication: ×400 magnification. $n = 4$ rats/group. *$P < 0.01$. *A* and *B*: significant differences in ECM expansion (arrows) were observed between animals injected with PCMV-TRH (*A*) versus PCMV-Con (*B*) (by Masson's trichrome). *C* and *D*: the LV TRH-overexpressing group (*C*) showed a significant increase in the degree of fibrosis (arrows) and the presence of profibrotic cells (myofibroblasts) in the cardiac interstitium compared with control animals (*D*) (by Sirius red). *E* and *F*: Azan-Mallory stain revealed large blue areas in PCMV-TRH-injected animals (*E*), indicating an increase in ECM expansion relative to PCMV-Con-injected animals (*F*). *G* and *H*: TRH immunostaining in both groups. Note the large area of positive brown immunostaining (arrows) in the PCMV-TRH group (*G*) compared with the PCMV-Con group (*H*). *I*: bars represent the quantification of ECM expansion by two stains (Masson's and Azan-Mallory), collagen (Sirius red), and TRH immunostained areas in both groups [PCMV-TRH (*A*) vs. PCMV-Con (*C*)].

the same animals such as the diencephalon, because it is known that the central TRH system participates not only in the endocrine system but also in cardiovascular regulation (6), as well as in the pancreas, where the tripeptide has also been found (19). No differences were observed between the two groups, indicating the specificity of the treatment in inducing LV TRH overexpression [diencephalic preproTRH: 100 ± 17 arbitrary units in the PCMV-Con group vs. 142 ± 22 arbitrary units in the PCMV-TRH group and pancreatic preproTRH: 100 ± 11 arbitrary units in the PCMV-Con group vs. 90 + 25 arbitrary units in the PCMV-TRH group, not significant (NS)]. Accordingly, at the end of the experiment, thyroid hormones were also evaluated in both groups of animals and no differences were found, showing the euthyroid status of the animals even with cardiac TRH overexpression (T_3: 1.14 ± 0.15 μg/dl in the PCMV-Con group vs. 1.25 ± 0.22 μg/dl in the PCMV-TRH group and T_4: 4.88 ± 0.35 ng/ml in the PCMV-Con group vs. 5.25 ± 0.42 ng/ml in the PCMV-TRH group, NS).

These results pointed out a specific activation of the TRH system in the LV of PCMV-TRH-injected rats.

Based on the TRH-induced profibrotic and prohypertrophic effects previously observed in SHRs, we evaluated fibrosis and ECM expansion with collagen type III expression, a protein related to the fibrosis process as an important component of the ECM. As hypothesized, there was a significant more than twofold increase ($P < 0.03$) of collagen type III expression in the LV TRH-overexpressing group with respect to the control group (Fig. 2*A*). This result was accompanied by significant increases in the expression of both recognized hypertrophic markers β-myosin heavy chain (β-MHC) and BNP compared with control Wistar rats injected with PCMV-Con ($P < 0.04$; Fig. 2*B*). Moreover, ANP expression was also increased, but the change did not reach statistical difference, in accordance with the literature, which pointed out BNP as a more sensitive marker for heart hypertrophy than ANP (data not shown). Again, we tested the rest of the heart chambers and found no changes either in the right ventricle or atria, although a tendency was observed in the expression of these markers in the septum in accordance with the small increase of TRH observed.

The PCMV-TRH effect on fibrotic and hypertrophic markers in the LV reinforces the concept that a single increase in the TRH system induces structural changes in a normal heart.

In addition, we evaluated morphometric variables and observed that LV TRH overexpression induced a slight but significant increase in cardiomyocyte diameter with respect to PCMV-Con-injected Wistar rats. Also, as reflected in cardiomyocyte diameter, the PCMV-TRH-injected group presented a significant decrease in myocyte density, evaluated as the number of cells per area, and in the number of capillaries, expressed as the number of capillaries per area. These results support the idea that LV TRH overexpression alone was able to provoke diverse structural changes in the normal heart. In line with these findings, similar changes in the interventricular septum were observed in the TRH-overexpressing group, suggesting that the plasmid reached most of the entire LV, as expected (Table 1).

During all experiments, SABP and body weight were measured weekly, and we did not find differences between the groups (SABP: 126 ± 11 mmHg in the PCMV-Con group vs. 130 ± 15 mmHg in the PCMV-TRH group and heart rate: 380 ± 22 beats/min in the PCMV-Con group vs. 355 ± 42 beats/min in the PCMV-TRH group, NS). Thus, it is unlikely that the structural changes observed could be consequence of an increase in arterial blood pressure.

At the end of the experiment, two additional hypertrophy indexes were measured (heart weight/body weight and heart weight/snout-tail long indexes), for which no significant differences were found. These normal ratios found in animals injected with PCMV-TRH were probably due to different magnitudes of the TRH effects on the fibrotic (strong) and hypertrophic (slight) processes. In addition, we cannot discard that the short period of the treatment (4 wk) was not sufficient to reveal ongoing macroscopic changes and that only microscopic changes were evident.

To further confirm the structural changes induced by LV TRH overexpression, histological experiments of the LV in

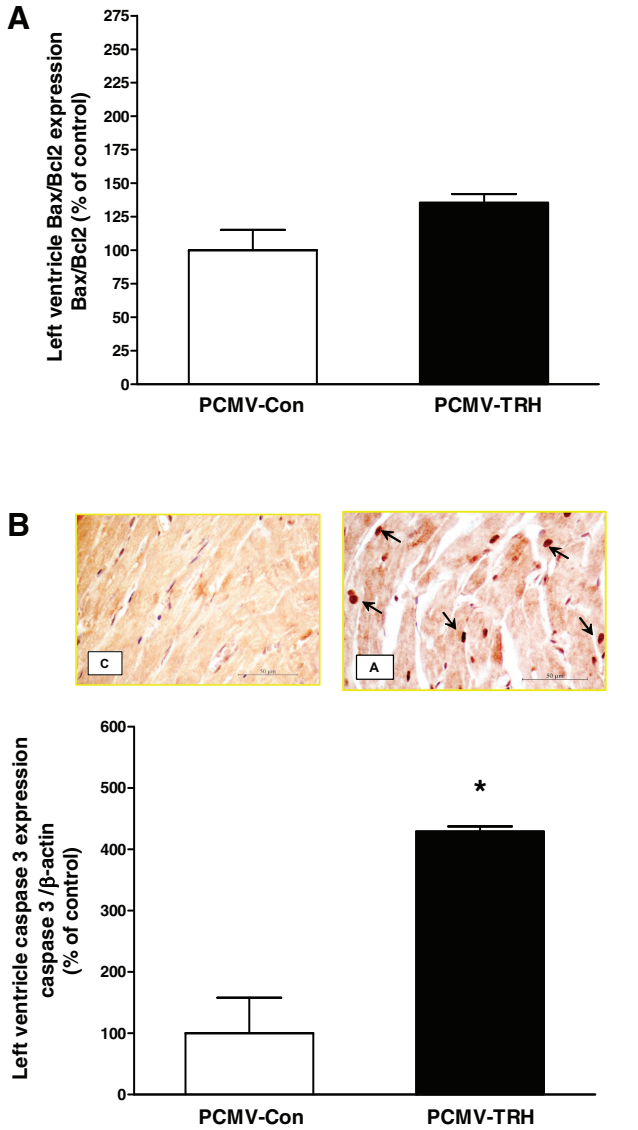

Fig. 4. Effect of LV TRH overexpression on apoptotic markers. mRNAs were determined by real-time PCR and normalized by β-actin expression. *A* and *B*: apoptotic ratio (Bax/Bcl-2; *A*) and caspase-3 mRNA expression with heart sections showing immunostaining for caspase-3 by the immunoperoxidase technique (*B*). Results are expressed as percentages of the control group. n = 6 rats/group. *$P < 0.05$ compared with the PCMV-Con group.

both groups were performed. As shown in Fig. 3, evaluation of ECM expansion was performed using Masson's trichrome (*A* and *B*) and Azan-Mallory staining (*E* and *F*). The results showed that LV TRH overexpression induced a significant and important ($P < 0.01$) increase in ECM expansion in normal Wistar rats. Furthermore, in agreement with the observation of elevated collagen type III mRNA expression in the LV in the TRH-overexpressing group, a substantial increase ($P < 0.01$) in collagen, as evaluated by Sirius red staining, was observed (Fig. 3, *C* and *D*).

Finally, in accordance with TRH mRNA expression and tripeptide content data, immunohistochemical analysis of the LV using a specific antibody against TRH (7) showed a positive brown signal in the PCMV-TRH group with respect to the PCMV-Con group (Fig. 3, *G* and *H*), confirming that in vivo overexpression was mainly induced in cardiomyocytes.

Since it is known that ECM expansion occurs after myocyte cell death, we hypothesized that PCMV-TRH group would present higher apoptotic indexes. In that sense, we evaluated apoptosis markers and measured the proapoptotic-to-antiapoptotic (Bax-to-Bcl-2) ratio (Fig. 4*A*), caspase-3 expression, and presence of caspase-3 by immunostaining (Fig. 4*B*). Although the Bax-to-Bcl-2 ratio did not show any differences between the two groups, we observed a significant ($P < 0.03$) increase in caspase-3 expression in the PCMV-TRH group. Indeed, we found an increase in the number of cells with positive staining for caspase-3 (by the immunoperoxidase technique) in the PCMV-TRH group (6.2 + 1.1 nuclei/area) with respect to the PCMV-Con group (0.6 + 0.7 nuclei/area, $P < 0.01$), indicating an apoptotic induction in these animals.

Finally, trying to examine which cell type was involved in TRH-induced effects, we tested TRH overexpression in primary Wistar rat cell cultures (myocyte and fibroblasts). We transitory transfected cardiac cells and measured TRH overexpression effects on gene expressions 24 and 96 h posttreatment.

As shown in Fig. 5*A*, TRH overexpression in myocytes was achieved 24 h after transfection, given that augmented TRH precursor expression was evident in the PCMV-TRH group. As expected, this overexpression was transitory, because 96 h after treatment, TRH precursor expression was similar to the control group. In agreement with the in vivo experiments, the TRH increase significantly induced the expression of BNP and β-MHC at 24 h (Fig. 5, *B* and *C*). Moreover, an induction of the apoptotic index (Bax/Bcl-2) was manifest at 24 h, when TRH overexpression was maximal.

Furthermore, as shown in myocytes, cardiac fibroblasts showed efficient TRH overexpression at 24 h posttransfection that provoked a significant increase in collagen type III expression 48 h after treatment, which was maximal at 96 h, the last time point studied (Fig. 6). These observations are in accordance with our knowledge of cardiac physiology, as myocyte death occurs first, followed by the ECM activation triggered by the collagen increase being fibrosis, the final stage of this process.

DISCUSSION

In 1992, a cardiac TRH system was reported, and, since then, many authors have studied the role of local TRH in cardiac physiology (1, 9, 26). An increase in TRH expression in the ventricle of infarcted rats after an 8-wk injury has been reported, suggesting the participation of the tripeptide in the cardiac damage (11). Afterward, our group showed that adult SHRs presented LV TRH system hyperactivity. We then dem-

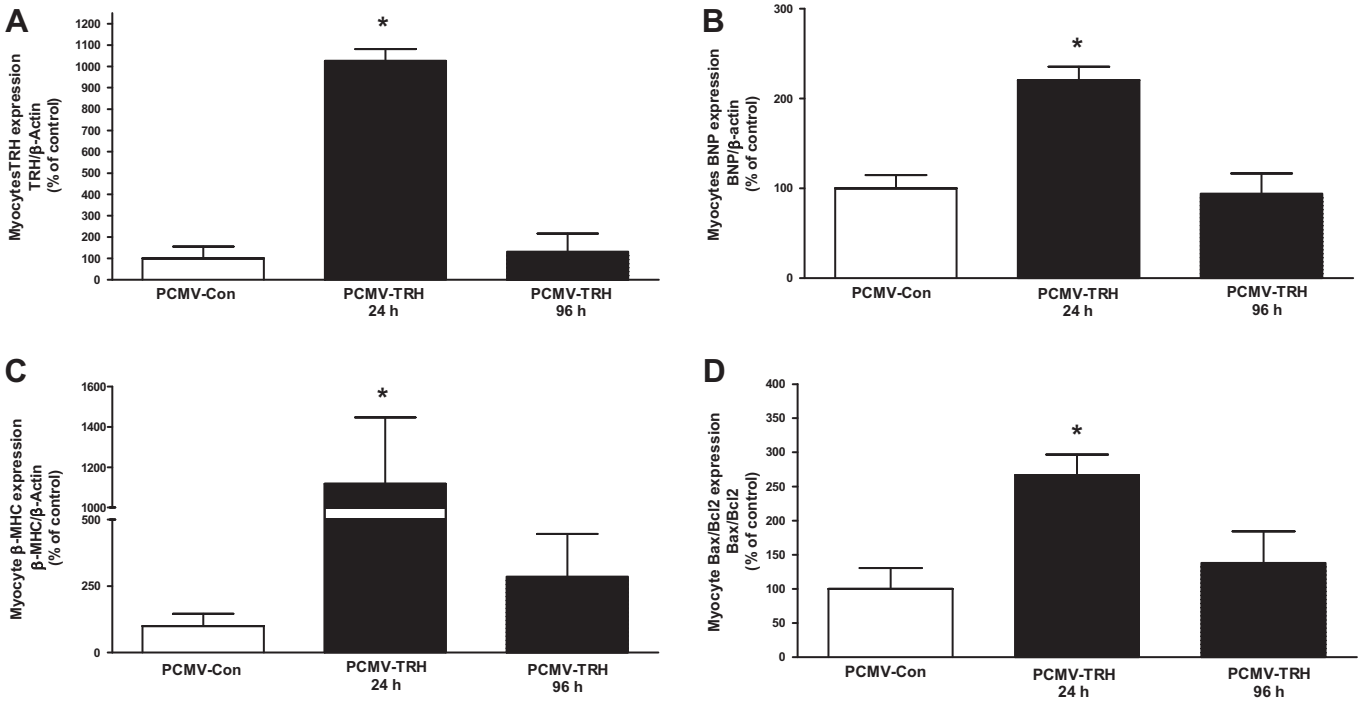

Fig. 5. Effect of TRH transfection on hypertrophic and apoptotic marker expression in myocyte primary cell cultures from Wistar neonates. mRNA expression was determined by real-time PCR and normalized by β-actin expression. *A–D*: TRH precursor expression (*A*), BNP expression (*B*), β-MHC expression (*C*), and apoptotic ratio (Bax/Bcl-2; *D*). Results are expressed as percentages of the control group. $n = 5$ rats/group. *$P < 0.05$, cells tranfected with PCMV-TRH compared with cells transfected with PCMV-Con.

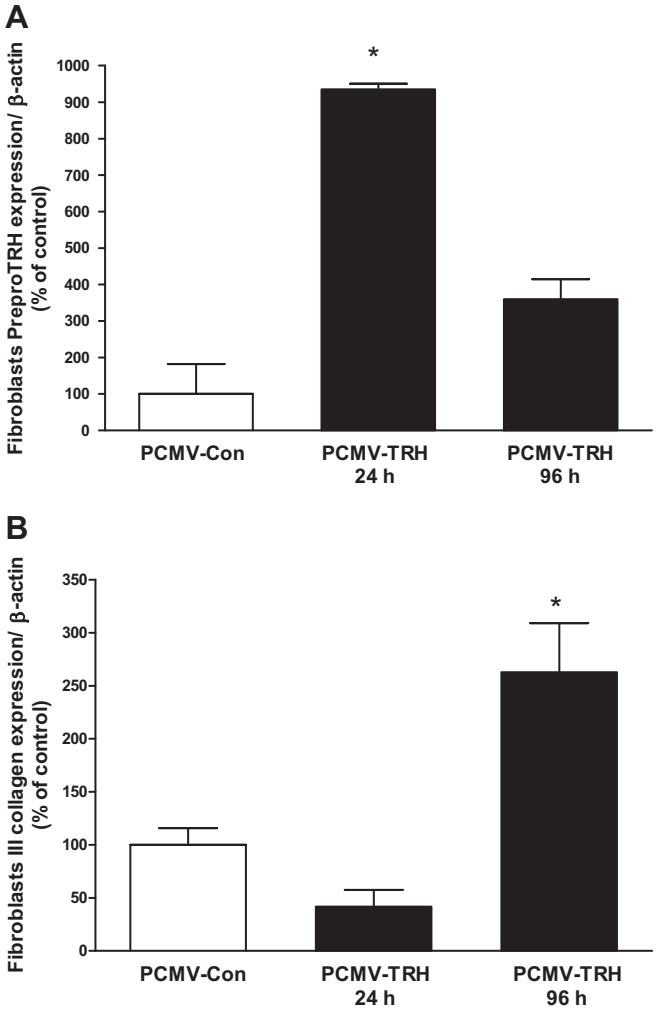

Fig. 6. Effect of TRH overexpression on collagen type III expression at 96 h after transfection in cardiac fibroblast primary cell cultures from Wistar neonates. mRNA expression was determined by real-time PCR and normalized by β-actin expression. *A* and *B*: TRH precursor expression (*A*) and collagen type III expression (*B*). Results are expressed as percentages of the control group. $n = 5$/group. *$P < 0.05$, cells tranfected with PCMV-TRH compared with cells transfected with PCMV-Con.

onstrated that long-term inhibition of the TRH system in young SHRs prevented cardiomyocyte enlargement, ECM expansion, and fibrosis development. Moreover, no differences were seen at the adult stage between Wistar-Kyoto rats and SHRs treated with specific TRH siRNA, indicating that the treatment was efficient and avoided detrimental TRH actions on cardiac tissue (23).

To our knowledge, all evidence on the role of cardiac TRH in heart damage has been obtained in systems where other abnormalities were concurrent (infarcted rats, SHRs, etc.) (11, 12). In this regard, the SHR model has been extensively studied, and the participation of many systems in LV hypertrophy development has been described (the renin-angiotensin system, endothelin system, adrenergic system, etc.) (2, 23, 18). On this basis, we speculated whether single ventricle TRH overexpression would be enough to induce cardiac structural changes in a normal rat independent of any other alteration.

LV TRH overexpression was efficiently induced in normal hearts of Wistar rats injected with PCMV-TRH compared with control rats injected with PCMV-Con. PCMV-TRH has been previously used to induce central TRH overexpression (8) and other proteins in cardiac tissue using the same technique. LV TRH overexpression was specific, as we did not find TRH expression changes in other tissues (brain and pancreas). Indeed, both groups showed similar plasma thyroid hormone levels, and the in vivo intracardiac treatment, which involved repeated injections of the plasmid, did not produce body weight or SABP changes throughout the experiment.

As expected, TRH overexpression for 4 wk was not enough to induce a 10-fold TRH increase, as has been observed in adults SHR (23); however, a twofold TRH increase was able to induce expression of fetal genes and fibrosis, suggesting that chronic LV TRH could lead to the development of fibrosis and hypertrophy in a normal rat.

In that sense, this long-term LV TRH overexpression induced an increase in the hypertrophy marker BNP that was accompanied by a smaller increase in ANP expression, indicating structural changes focused to a hypertrophic phenotype shift of the heart tissue. Accordingly, we found a significant increase in β-MHC and collagen type III expression that was confirmed by histological experiments in which an increase in myocyte diameter and ECM protein expression were observed only in the LV in the TRH-overexpressing group.

On the other hand, many authors have described that the first structural changes, which include a reduction in myocyte mass and associated functional loss, are due to increased cell death by apoptosis (14, 21). Therefore, increases in proapoptotic Bax and caspase-3 proteins have been described in hypertrophied LVs of young and adult SHRs, and, as we have shown in previous work, long-term inhibition of LV TRH ameliorates not only heart hypertrophy but also reduces the exaggerated apoptosis that accompanies heart failure in this model (23). On this basis, as a first step, we evaluated the apoptosis ratio (Bax/Bcl-2) as an index of cells' susceptibility to apoptosis and measured caspase-3 RNA and protein levels. We found a significant increase ($P < 0.03$) in caspase-3 expression only in the PCMV-TRH-injected group, although Bax/Bcl-2 did not show any difference at the end of the experiment. Moreover, we evaluated the caspase-3 signal by an immunoperoxidase technique and found a significant increase in caspase-3 in the TRH-overexpressing group, confirming the increased activity of a caspase-3-mediated apoptotic pathway in these hearts.

Our results are the first demonstration that, in a normal heart, the increase in LV TRH expression alone could induce structural changes that are shared with features of hypertrophy. These observations confirm that cardiac TRH induces a heart shift, in which structural alterations such as fibrosis, hypertrophy, and apoptosis could be involved, independently of any other system alterations.

Finally, we developed experiments using primary cardiac cell cultures to try to confirm TRH-induced effects in vitro. As expected, TRH overexpression was successfully induced in both cell types 24 h after plasmid transfection, confirming the presence of TRH biosynthesis machinery in either myocytes or fibroblasts. We extended the experiment up to 96 h, when the acute TRH overexpression effect was negligible. The difference in the time course with the in vivo experiment is probably due to the experimental procedure (transfection cell efficiency, cell division, etc.), as discussed below.

However, as hypothesized, TRH overexpression was capable to induce expression of hypertrophic markers BNP and β-MHC, effects that reach a peak at 24 h.

In agreement with the notion that hypertrophy is accompanied by myocyte cell death, we found increased apoptotic index expression at 24 h, when TRH overexpression reached its maximum, indicating that TRH overexpression in myocyte cells is involved in hypertrophic and apoptotic processes.

In parallel, we studied the effects of TRH overexpression in cardiac fibroblasts, and, unlike what we observed in myocytes, TRH induction triggered a significant increased in collagen type III expression that was maximal (5-fold) at 96 h, indicating that TRH participates in ECM expansion the latest stage.

It is well known that primary cell culture transfection did not respond as in vivo transfection; indeed, we found discrepancies between TRH overexpression, i.e., induced effect time course, limiting the comparison between in vitro and in vivo results. Nevertheless, as a whole, the cell culture results confirmed a TRH direct action on cardiac cells.

Finally, both in vitro and in vivo results were in agreement with our previous publication (23), in which disruption of the cardiac TRH system impeded fibrosis and hypertrophy in SHRs, and outstand the contribution of the TRH system in the structural changes that occur during cardiac pathologies, although further studies would be necessary to define the cellular mechanism.

Interestingly, several cardiac pathologies have shown increased cardiac fibrosis, with ECM expansion, myocyte hypertrophy, and apoptosis; examples include infarct and obesity with hyperleptinemia, where TRH hyperactivity has been observed (13, 5). In these situations where the cardiac TRH system is upregulated, it appears that its inhibition may be protective for the heart.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.L.S., L.S.P.D., J.E.T., G.C., A.L.A., and S.I.G. performed experiments; M.L.S., M.S.L., and S.I.G. analyzed data; M.S.L., S.F., and S.I.G. interpreted results of experiments; S.F., C.J.P., and S.I.G. edited and revised manuscript; S.I.G. conception and design of research; S.I.G. prepared figures; S.I.G. drafted manuscript; S.I.G. approved final version of manuscript.

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