Heart

Cardiac Thyrotropin-Releasing Hormone Mediates Left Ventricular Hypertrophy in Spontaneously Hypertensive Rats

Mariano L. Schuman, Maria S. Landa, Jorge E. Toblli, Ludmila S. Peres Diaz, Azucena L. Alvarez, Samuel Finkielman, Leonardo Paz, Gabriel Cao, Carlos J. Pirola, Silvia I. García

See Editorial Commentary, pp 26–28

Abstract—Local thyrotropin-releasing hormone (TRH) may be involved in cardiac pathophysiology, but its role in left ventricular hypertrophy (LVH) is still unknown. We studied whether local TRH is involved in LVH of spontaneously hypertensive rats (SHR) by investigating TRH expression and its long-term inhibition by interference RNA (TRH-iRNA) during LVH development at 2 stages (prehypertrophy and hypertrophy). SHR and their control rats (WKY) were compared. Cardiac hypertrophy was expressed as heart/total body weight (HW/BW) ratio. TRH content (radioimmuno assay), preproTRH, TRH receptor type I, brain natriuretic peptide (BNP), and collagen mRNA expressions (real-time polymerase chain reaction) were measured. For long-term inhibition of TRH, TRH-iRNA was injected into the left ventricle (LV) wall for 8 weeks. Hearts were processed for morphometric studies and immunohistochemical analysis using antibodies against α-smooth muscle actin and collagen type III. LV preproTRH-mRNA abundance was similar in both strains at 7 weeks of age. At the hypertrophic stage (18 weeks old), however, there was a 15-fold increase in SHR versus WKY, consistent with a significant increase in tripeptide levels and the expression of its receptor. Specific LV-TRH inhibition at the prehypertensive stage with TRH-iRNA, which decreased >50% preproTRH expression and tripeptide levels, prevented LVH development as shown by the normal HW/BW ratio observed in TRH-iRNA–treated SHR. In addition, TRH-iRNA impeded the increase in BNP and type III collagen expressions and prevented the increase in cardiomyocyte diameter evident in mismatch iRNA-treated adult SHR. These results show for the first time that the cardiac TRH system is involved in the development of LVH in SHR. (Hypertension. 2011;57:103-109.)

Key Words: TRH ▪ cardiac hypertrophy ▪ rat ▪ SHR ▪ interference RNA

Thyrotropin-releasing hormone (TRH), a small neuropeptide (p-Glu-His-Pro-NH2) initially identified in the hypothalamus, is amply distributed in the central nervous system and in other extraneural tissues and has been shown to have central and peripheral biological effects independent of thyroid hormone production. TRH also acts on the cardiovascular system of rodents.4,5

Many groups have identified preproTRH-mRNA by Northern blot analysis and RNAse protection assay in rat cardiac tissues and have referred the presence of specific type I TRH receptors (TRH-R1) in ventricles, establishing that a TRH system is present in the rat heart.6–8 In contrast to the hypothalamic TRH system, cardiac preproTRH-mRNA may be augmented by glucocorticoids and by testosterone but may not be regulated by T3.8 In addition, Hasegawa et al9 reported for the first time an inotropic effect of TRH on the guinea pig myocardium, implying that this effect could be mediated by an increase in a slow inward Ca2+ current. Furthermore, Socci et al7 found similar results and reported that TRH modulates cardiac contractility of isolated rat hearts as an autocrine factor in a concentration-dependent manner, involving more than one TRH receptor. Enhanced ventricular contractility by TRH was also demonstrated by other authors in both open-chested dog preparation and ex vivo ventricular myocytes, using video edge cinematography.8

More recently, using DNA microarrays, Jin et al10 identified preproTRH as one of the genes induced in the left ventricle (LV) of rats with heart failure, 8 weeks after...
myocardial infarction. They also suggested that TRH can increase cardiac performance in rats with ischemic cardiomyopathy.\textsuperscript{11} Based on these results, TRH was postulated to play an autocrine or paracrine role in cardiac physiology, although the physiological role of endogenous TRH in the heart remains unknown. Moreover, to our knowledge, no evidence has been reported on the role of the cardiac TRH system on cardiac hypertrophy.

In this study, spontaneously hypertensive rats (SHR) were used as a cardiac hypertrophy model to investigate the role of the LV cardiac TRH system in the development of left ventricular hypertrophy (LVH).

We show, for the first time, the expression of TRH system components in prehypertrophic and hypertrophied LVs of SHR and developed a long-term inhibition of the LV TRH system to evaluate the participation of the local TRH system in the development of LVH.

### Methods

All reagents were from Sigma unless indicated. The Institutional Animal Care and Use Committee approved the animal experimentation protocols following the ethical guidelines.

### Animals

Male SHR and WKY rats (Charles River Laboratories, Wilmington, MA) (10 per group) at 7 weeks old and at the hypertensive stages (18 weeks old) were used. The animals were housed in a room with controlled temperature (23 ± 1°C) under a 12-hour light/dark schedule. Before the experiments, blood pressure was recorded.

**In Vivo Interference RNA Treatment**

Seven-week-old SHR were assigned in a random blind fashion to 1 of the 2 groups, (1) specific TRH-interference RNA (TRH-iRNA) and (2) scramble mismatched control (Con-iRNA) (n = 10 per group), and were anesthetized (90 mg/kg ketamine and 10 mg/kg xylazine). Injections were conducted every 2 weeks during the experiment (8 weeks) to maintain the knockdown effect. All injections were done under echography.

For a detailed Materials and Methods, please see the online Data Supplement, available at http://hyper.ahajournals.org.

### Results

**LV-TRH Hyperactivity in the Hypertrophied Ventricle**

We used SHR at 2 different ages and their controls, age-matched WKY. At the age of 7 weeks the SHR presented a small increase in blood pressure without any difference in the heart/total body weight (HW/BW) ratio with respect to the normotensive strain, in comparison with adult SHR (18 weeks old), which were hypertensive and showed a markedly hypertrophied heart with a significant increase in the HW/BW ratio (Figure 1). As we had hypothesized, we found an age-dependent and significant increase in TRH expression in the LV of SHR with respect to the normotensive and normotrophic WKY rats, as TRH content increased 6-fold at 18 weeks when LVH was marked (Figure 2A). In contrast, at the prehypertrophic stage, no significant changes between both strains were observed. As changes in peptide content could reflect both differential posttranslational processing and expression, we also evaluated preproTRH-mRNA abundance. The increase in TRH content at the hypertrophic stage was accompanied by a significant increase in the preproTRH-mRNA abundance in the SHR LV (Figure 2B) compared to their age-matched WKY controls (P < 0.01). In parallel with these results, we also found a significant increase in the specific type 1 receptor (TRH-R1) gene expression compared to the 7-week-old SHR; a smaller increase was also seen when compared to the age-matched WKY rats, although it did not reach statistical significance. Peptide, precursor, and specific receptor results showed a hyperactivity of the TRH system in the hypertrophied ventricle. No significant changes were seen in the WKY strain at both stages.

SHR LV changes seem to be specific since no changes were found in the right ventricle at this age either in TRH content (18 weeks WKY 0.6 ± 0.1 pg/mg protein, 18 weeks SHR 0.55 ± 0.2 pg/mg protein) or preproTRH-mRNA abundance (18-week WKY 5.7 ± 0.5 arbitrary units, 18-week SHR 6.55 ± 0.5 arbitrary units). We observed a slight but not significant increase in TRH content in the septum of adult SHR (18-week WKY 0.35 ± 0.1 pg/mg protein, 18-week SHR 0.47 ± 0.15 pg/mg protein) that was, accordingly, similar to the septum preproTRH-mRNA abundance found between both strains (18-week WKY 6.3 ± 0.35 arbitrary units, 18-week SHR 5.85 ± 0.42 arbitrary units).

These results pointed out an activation of the TRH system on the hypertrophied LV of SHR.

**LV-TRH Long-Term Inhibition by Intracardiac iRNA Treatment**

First, we evaluated the efficacy of the specific TRH-iRNA treatment, and we measured preproTRH-mRNA abundance and TRH content in the LV of the SHR injected with the specific TRH-iRNA or the mismatched Con-iRNA oligonu-
cleotides. As shown in Figure 3A (left), the inhibition of TRH by specific iRNA treatment (SHR+TRH-iRNA) decreased LV preproTRH gene expression by 53% compared to the same strain treated with control oligos (SHR+Con-iRNA).

A tendency of decrease was observed in the septum of the atria or right ventricle. As shown in Figure 3B, the specific and long-lasting inhibition of LV-TRH expression impeded the expected increase of the hypertrophy index in adult SHR (18 weeks old). Moreover, the TRH-iRNA–treated SHR presented a HW/BW ratio similar to that of control normotensive WKY rats, indicating the normotrophic stage of the treated SHR (Figure 3B, left). These results were confirmed using the HW/snout-tail long index; again (Figure 3B, right), WKY and TRH-iRNA–treated SHR showed similar indices that were significantly lower compared to those of the Con-iRNA–treated SHR.

These normal ratios found in the animals with the inhibition of the LV TRH were not caused by normalization of blood pressure or changes in the body weight, as we measured both variables weekly during the entire experiment, and we did not find any significant changes. (Figure 3C).

In addition, we characterized the effect of the long-lasting inhibition of LV TRH on molecular expression of genes previously shown to be involved in the progression and widely recognized as markers of cardiac hypertrophy, such as brain natriuretic peptide (BNP) and another involved in fibrosis development, the type III collagen, in both groups of hypertensive treated rats. As observed in Figure 4A, the group of SHR with LV-TRH inhibition showed a 55% decrease in type III collagen expression compared to control mismatched iRNA–treated SHR. In accordance, a significant reduction in BNP expression was observed in the same group compared to control mismatch-iRNA–treated SHR. Together, these results suggest that the specific TRH inhibition in the LV may be protective for the heart.

To confirm the specificity of the in vivo iRNA treatment, we decided to quantify preproTRH gene expression on the diencephalon, because it is known that the TRH system participates not only in the endocrine system but also in cardiovascular regulation and in other extraneuronal tissues where the tripeptide has been found.13 We also evaluated the thyroid status in both groups of animals. As shown in Figure 4B, cardiac iRNA treatment against TRH did not alter either the diencephalic preproTRH gene expression (right) or plasma thyroid hormone levels (left) in any group. In addition, no differences between the 2 groups were seen in pancreas and liver (data not shown), indicating that the treatment was tissue specific and its effects were independent of the thyroid system.

To further confirm the reversion of LVH by TRH inhibition, histological studies of LV in both groups were performed. The specific TRH-iRNA treatment prevents the significant increase in the cardiomyocyte diameter, which was expected and evident only in the SHR treated with the scrambled control iRNA, as shown in the supplemental table.

According to the cardiomyocyte diameter, TRH-iRNA–treated SHR present a significant increase in myocyte density, evaluated as the number of cells per area and as the number of capillary per area, indicating that LV-TRH inhibition prevents hypertrophy development in SHR. In line with these findings, similar changes in the interventricular septum were observed in the SHR+TRH-iRNA group, suggesting that the TRH-iRNA reach the entire LV, as was expected.

As illustrated in Figure 5, evaluation of extracellular matrix (ECM) expansion by Masson’s trichome (A, B) and Sirius red (C, D) stains showed that LV-TRH inhibition prevents (P<0.01) the increase in ECM expected for an adult SHR. Furthermore, LV-TRH inhibition prevents the increase of α-smooth muscle actin, a protein related to the fibrosis...
process and expressed in interstitial myofibroblasts as shown in Figure 5E and 5F.

In favor of a better conserved cardiac structure in the LV-TRH–inhibited group and in agreement with the observation of 55% less type III collagen mRNA expression, a substantial reduction ($P<0.01$) in collagen III immunostaining in the LV-TRH–inhibited SHR group with respect to the control SHR was observed (Figure 5G and 5H).

Finally, Figure 5I shows bars representing the quantification of ECM expansion, -smooth muscle actin, and collagen III immunostaining areas in both groups of animals.

**Discussion**

Many groups have defined SHR as a good model for human cardiac pathophysiology, in particular LVH. Since 1992, when a cardiac TRH system was reported, many authors have been studying the role of local TRH in cardiac physiology. An increase in TRH expression in the ventricle of infarct rats after an 8-week injury has been reported, suggesting the participation of the tripeptide in the cardiac damage. Thus, the aim of this study was to evaluate the role of the cardiac TRH system in the LVH of SHR. To further characterize the changes in the TRH system during cardiac hypertrophy development, we examined SHR at 2 stages, at 7 and at 18 weeks of age, to encompass the heart shift from normotrophic to hypertrophic phenotype. As expected, no changes either in TRH content or in preproTRH-mRNA abundance were found at 7 weeks when both hypertension and hypertrophy were absent, as seen by comparing normotensive with respect to hypertensive strain, showing that alterations attributed only to genetic differences between strains were unlikely.

Interestingly, in accordance with our hypothesis, we found a marked increase in the expression of the cardiac TRH system (preproTRH-mRNA, peptide, and its specific receptor) in the hypertrophied LV, as confirmed by the significant increase in BNP expression and the hypertrophy indices used.
These data suggest the possible participation of the cardiac LV TRH in this pathology either as a promoting or a protective factor.

The association of cardiac TRH with hypertrophy development is supported by our findings that TRH peptide, its precursor, and its specific receptor are upregulated only on the hypertrophied LV as no changes were seen in other cardiac tissue (right ventricle or septum).

To our knowledge, this is the first description of hyperactivity of the cardiac TRH system in the hypertrophied LV of adult SHR, indicating that this hyperactivity is associated with the development of the hypertrophy.

As there is no commercial TRH antagonist available, to further investigate the TRH role in this model, we developed a long-term inhibition of the TRH system in the LV of adult SHR by using iRNA against the preproTRH gene and demonstrated that the reduction of the augmented LV-TRH expression in SHR impedes LVH development, showing for the first time that LV TRH promotes hypertrophy in this model.

In fact, specific TRH-iRNA treatment that normalizes the augmented TRH-LV expression prevents the increase of the 2 hypertrophy ratios expected in adult SHR. Moreover, no differences were seen between the normal WKY rats and the SHR treated with the specific TRH-iRNA, indicating that the treatment was efficient and avoids detrimental TRH actions on cardiac tissue. This reduction in the indices were not caused by differences in body weight as the animals did not show any body weight difference along the treatment between groups. Despite the fact that systolic blood pressure levels remained high in both groups of hypertensive rats, TRH-iRNA normalized LVH even when overload was maintained. These results suggest that the group in which hypertrophy development was avoided would develop a more severe cardiac failure, as it has been described that in a first phase, LV hypertrophy helps the heart to function with a higher requirement. However, additional investigations will be necessary as we did not follow the protocol for more than 8 weeks.

In accordance with pathology results, we were able to observe that LV-TRH inhibition impedes not only the increase of the hypertrophy marker gene expression BNP but also collagen III expression and content, indicating that hypertrophy and fibrosis development was impeded at the level characterized by these sensitive markers despite the high blood pressure.

The normal levels of BNP expression in the LV of TRH-iRNA–treated SHR was accompanied by a reduced atrial natriuretic peptide expression (data not shown), indicating a regression of the hypertrophied tissue to the fetal gene expression program. Finally, pathology studies were able to confirm the benefit of the specific TRH-iRNA
treatment that prevents the increase in the myocyte diameter and the ECM protein expression. These results are consistent with the prevention of hypertrophy ratio increases in the TRH-iRNA–treated group.

Additionally, adult SHR treated with the specific TRH-iRNA showed absence of fibrosis in compared to the SHR treated with mismatch-iRNA, as we observed with the immunostaining for collagen III and Sirius red techniques.

iRNA has been shown to be efficient for specific gene silencing\textsuperscript{16}; indeed, potential effects on other tissues may not be significant, as we were able to show that effects were independent of central TRH and thyroid status.

Although this in vivo intracardiac treatment, which involved repeated injections of naked iRNA, is not feasible as therapy, these results show a useful tool to identify novel gene functions particular in the heart in rodents and also suggest that intracardiac iRNA injections may be suitable for long-term treatment. In fact, we demonstrate for the first time that repeated intracardiac injections of a specific iRNA could reduce the expression of the target gene.

The results in the present study demonstrate the following: (1) there was TRH hyperactivity in the hypertrophied LV of the SHR, and (2) an in vivo specific TRH-iRNA treatment induced downregulation of LV-TRH production, preventing cardiac hypertrophy and fibrosis development.

These findings suggest new potential approaches for heart disease, as we demonstrated that TRH is involved in cardiac hypertrophy and is associated with the hypertrophic and fibrotic process, effects that can be disrupted by targeting TRH.

**Perspectives**

Our results indicated that the tripeptide may induce heart damage, where hypertrophy, fibrosis, and apoptosis could be involved. Interestingly, an increased cell death occurs before ABP increase in the SHR heart, as reported by Liu et al.\textsuperscript{17}

Additional studies using cardiac cell cultures, as we found specific type I TRH receptor expression in cultured neonatal cardiomyocytes and fibroblasts (unpublished results), would be necessary to elucidate the mechanism by which TRH induces the development of LVH.

**Sources of Funding**

This work was supported by grant PICT 03-13862 from the Agencia Nacional de Promocion Cientifica y Tecnol\`ogica (ANPCyT) and grant PIP 11220090100636 from the National Council of Research and Technology (CONICET).

**Disclosures**

None.

**References**

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Hypertension. 2011;57:103-109; originally published online December 6, 2010;
doi: 10.1161/HYPERTENSIONAHA.110.161265

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/57/1/103

Data Supplement (unedited) at:
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Cardiac Thyrotropin Releasing Hormone (TRH) Mediates Left Ventricular Hypertrophy in Spontaneously Hypertensive Rats (SHR).

Mariano L Schuman, PhD1; Maria S Landa, PhD1,2; Jorge E Toblli, PhD,MD3; Ludmila S Peres Diaz1; Azucena L Alvarez2; Samuel Finkielman, MD1; Leonardo Paz, MD3; Gabriel Cao, MD4; CJ Pirola, PhD2 and Silvia I García, PhD1.

Departamento de Cardiología Molecular1, Departamento de Genética y Biología Molecular de Enfermedades Complejas2 and Servicio de Anatomía Patológica3 Instituto de Investigaciones Médicas Alfredo Lanari, Universidad de Buenos Aires, Buenos Aires, Argentina; IDIM-CONICET; Laboratory of Experimental Medicine, Hospital Alemán, Buenos Aires, Argentina4.

“TRH and Cardiac Hypertrophy in SHR”

Correspondence Author:
Silvia I. García, PhD.
Departamento de Cardiología Molecular,
Instituto de Investigaciones Médicas Alfredo Lanari, UBA
IDIM-CONICET
Materials and Methods

In vivo iRNA treatment:

Previously, we performed a dose-curve (20, 40, and 100 µg iRNA per injection), and as we found the maximal downregulation effect at 40 µg we decided to use it as unique dose. Forty µg iRNA/100 µL saline were injected in the left ventricle (LV) wall. All injections were done under echography, the chests of the rats were shaved and cleaned. Gel was liberally applied to the exterior body surface before the 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 divided in three different sites (from base to apex) to surround the entire LV. iRNA oligonucleotides were dissolved in saline. Systolic blood pressure (SBP) by the plethysmography-tail method and body weight were measured weekly. All experiments were also performed blindly with respect to the treatment.

Specific stealth iRNAs from Invitrogen (CA, USA) were used, which present a chemical modification resulting in lower toxicity and higher stability. The Block-iT ™ iRNA Designer software for the iRNA selection was used, and a mix of two double-strand sequences against preproTRH (TRH-iRNA 498 and TRH-iRNA 138) and a mix of two control iRNA sequences carrying mismatches compared with the specific ones (Con-iRNA 498 and Con-iRNA 138) were used as follows:

TRH-iRNA 498: 5’ACGGUCCUGGUUACCAGAUUUCUUU3’ and AS :5’AAAGAAA UCUGUAACCAGGACCGU 3’;
TRH-iRNA 138: 5’ GAUCUUACAACCUAUCUGUAUCCCU 3’ and AS : 5’AGGGAUACCAGUAGGGUGAAGAUC 3’
Con-iRNA 498: 5’ACGCCGUUGAUCCGAUAUUUCUUU 3’ and AS : 5’AAACAGAUAUCGGAUCAACGCGGCU 3’;
Con-iRNA 138: 5’GAUCACUAUCCGUCAUAUGUCUCCCU 3’ and AS :5’AGGGAGCAUAUGACGGAUAGUGAUC 3’

The screening of known rat genes from genomic databases of the National Center for Biological Information using the Blast program indicates specificity of the sequence used in the iRNA design and confirms their 100% homology with rat preproTRH gene. The observed effects are not due to a nonspecific toxicity because the survival of animals during the treatment was 100% and no sign of toxicity after decapitation and morphology analysis was observed.

TRH content:

For peptide extraction, cardiac tissue samples were collected in acetic acid 2 mol/L, HCl 0.1 mol/L. All samples were boiled for 20 min and centrifuged at 10000 rpm, and the supernatant was lyophilized. The residue was dissolved in the appropriate buffer for measurement of TRH-like immunoreactivity by RIA or the mobile phase for the identification of authentic TRH by high performance liquid chromatography. This procedure and the RIA for TRH have been reported in detail 1. Briefly, the cross-reactivity of the antiserum was 100% for p-Glu-His-Pro-NH2 (TRH), 15% for 3-methyl-TRH, 2.5%
for Glu-TRH, 0.03% for p-Glu-Glu-Pro-NH₂, 0.02% for TRH-OH, and <0.002% for Glu, His, Pro and cyclo(Hys-Pro). Standards or samples were incubated with ¹²⁵I-TRH and anti-TRH (1/8000) at 4°C overnight. The free hormone was pelleted using Carbon Dextran T-70. All samples were assayed in duplicate. The minimum detectable amount was 5 pg. Intra- and inter-assay coefficients of variation were less than 7.0 and 10.0 %, respectively, along the standard curve concentrations used.

Quantitative real-time PCR:
Quantification of preproTRH, BNP, type III collagen, and TRH-RI mRNA expression was performed using a real-time RT-PCR technique normalized by the β-actin housekeeping gene expression. Briefly, for cDNA synthesis, 2 µg of total RNA and 1 µg of random hexamer primers (Promega Corp, WI, USA) were heated at 70°C for 5 min, followed by incubation at 4°C. After adding 5 µL of M-MLV RT 5X buffer (250 mmol/L Tris-HCl, pH 8.3, 375 mmol/L KCl, 15 mmol/L MgCl₂, 50 mmol/L DTT), 25 U RNasin (Promega Corp, WI, USA), 1.25 µL of 10 mmol/L dNTPs (Invitrogen, CA, USA), and 200 U of MMLV-reverse transcriptase (Promega Corp, WI, USA), the reaction was carried out at 37°C for 1 h, followed by inactivation of the enzyme at 95°C for 10 min.

Real-time PCR was performed using a BioRad iQ iCycler Detection System (BioRad Laboratories, Ltd, CA, USA) with SYBER green fluorophore. Reactions (duplicates) were carried out in a total volume of 20 µL. A 3-step protocol (95°C for 30 sec, 60°C, 64°C or 66°C annealing for 30 sec, 72°C for 40 sec) was repeated for 40 cycles. Rat (r) primers (Invitrogen, CA, USA) were designed as follows:

r-preproTRH:F:5´GGTGCGTCCTTAGACTCCTG3´; R:5´CTCCTCACTGCCCATGAAT3´;
r-BNP: F:5´ATCCACGATGCAGAAGCTG3´; R:5´AGGCCTGTCTTTGAGACCTA3´;
r-CollgIII: F:5´GTCCAGGGATACGGGGTATG3´; R:5´CAGATGGGCCAGGAGGAC3´;
r-ß-actin: F:5´TTCCTGGGTATGGAATCCTG3´; R:5´CAGCAATGCCTGGGTACAT3´;
r-Trh-RI: F:5´AAGCAGGTCAACCCAGATGCT3´; R:5´GTAAATCACCAGGGTTGGATGG3´

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

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. Three-micron sections were cut and stained with hematoxylin-eosin (H&E), Masson’s Trichrome, and Sirius red. All observations in light microscopy were performed using a Nikon E400 light microscope (Nikon Instrument Group, Melville, New York, USA).

Immunohistochemical studies:
Immunolabeling of specimens was carried out using a modified avidin-biotin-peroxidase technique (Vectastain ABC kit, Universal Elite, Vector Laboratories, California, USA). Following deparaffinization and rehydration, the sections were washed in phosphate buffer
saline (PBS) for 5 min. Quenching of endogenous peroxidase activity was achieved by incubating the sections for 30 min in 1% hydrogen peroxide in methanol. After washing in PBS (pH 7.2) for 20 min, the sections were incubated with blocking serum for a further 20 min. Thereafter, the sections were rinsed in PBS and incubated with biotynilated universal antibody for 30 min. After washing in PBS a final time, the sections were incubated for 40 min with Vectastain Elite ABC reagent (Vector Laboratories, California, USA) and exposed for 5 min to 0.1% diaminobenzidine (Polyscience, Warrington, Pennsylvania, USA) and 0.2% hydrogen peroxide in 50 mmol/L Tris buffer (pH 8). For the purpose of evaluating the process of fibrosis, an anti-mouse α-smooth muscle actin monoclonal antibody (sc-130617 Santa Cruz Biotechnology, Inc. CA) and a monoclonal antibody anti-collagen type III (Biogenex, San Roman, CA), both at a dilution of 1:100, were used.

Morphological and quantitation analysis
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, LP, Silver Spring, Maryland, USA). Histomorphometric evaluation of heart was carried out according to the following schedule: extracellular matrix (ECM) expansion, numerical density of myocytes and coronary capillaries (vessels < 8µ) within the confines of each of the 20 random viewed at 400x magnification. In every heart, positive immunostaining for α-smooth-muscle actin and collagen III was assessed and the data were expressed as percentage per area.

Biochemical and pathology studies:
At the end of the treatment, the rats were weighted and the snout-tail long index was measured before the animals were sacrificed by decapitation. Hearts were rapidly removed and weighted. In some animals, cardiac tissues were separated (atria, left ventricle, right ventricle, and septum) for TRH determination by RIA and for quantification of preproTRH, brain natriuretic peptide (BNP), type III collagen, and TRH-R1 mRNA expression by real-time RT-PCR; others were used for pathology studies. LVH indexes were expressed as heart to total body weight (HW/BW) and heart to snout-tail long-ratios.

Statistics:
Values were expressed as mean ± SEM. All statistical analyses were performed using absolute values and processed through Statistics, version 6.0 (Software, Inc, California, USA). The assumption test to determine the Gaussian distribution was performed by the Kolmogorov–Smirnov method. For parameters with Gaussian distribution, comparisons between groups were carried out using one-way analysis of variance (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 t-test or Mann-Whitney test when appropriate. A p value of less than 0.05 was considered significant.

References:
Table S1

<table>
<thead>
<tr>
<th>Morphometric Variables</th>
<th>SHR+Con-iRNA</th>
<th>SHR+TRH-iRNA</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVW cardiomyocyte diameter (µm)</td>
<td>37.7 ± 0.9</td>
<td>31.2 ± 0.7</td>
<td>&lt; 0.01</td>
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<tr>
<td>LVW cardiomyocyte density (number of cardiomyocyte /area)</td>
<td>5.6 ± 0.4</td>
<td>9.8 ± 1.0</td>
<td>&lt; 0.01</td>
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<tr>
<td>LVW number of capillaries /area</td>
<td>11.7 ± 0.6</td>
<td>21.1 ± 1.1</td>
<td>&lt; 0.01</td>
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<tr>
<td>LVW myocyte / capillary ratio</td>
<td>0.48 ± 0.03</td>
<td>0.46 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>IVS cardiomyocyte diameter (µm)</td>
<td>36.3 ± 1.2</td>
<td>29.9 ± 0.8</td>
<td>&lt; 0.01</td>
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<tr>
<td>IVS cardiomyocyte density (number of cardiomyocyte /area)</td>
<td>6.1 ± 0.6</td>
<td>11.0 ± 1.0</td>
<td>&lt; 0.01</td>
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<td>IVS number of capillaries /area</td>
<td>12.2 ± 0.7</td>
<td>20.6 ± 0.8</td>
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<tr>
<td>IVS myocyte / capillary ratio</td>
<td>0.51 ± 0.06</td>
<td>0.54 ± 0.06</td>
<td>NS</td>
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</table>

LVW: left ventricular wall
IVS: interventricular septum