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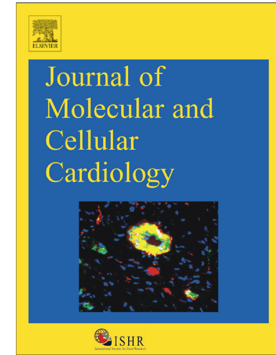

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Angiotensin II Requires an Intact Cardiac Thyrotropin-Releasing Hormone (TRH) System to Induce Cardiac Hypertrophy in Mouse.

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Abstract

Cardiac tyhrotropin-releasing hormone (TRH) is overexpressed in the hypertrophied left ventricle (LV) of spontaneously hypertensive rats (SHR) and its inhibition prevents both hypertrophy and fibrosis. In a normal heart, the TRH increase induces fibrosis and hypertrophy opening the question of whether TRH could be a common mediator of left ventricular hypertrophy (LVH). We used angiotensin II (AngII) as an inductor of LVH to evaluate if the blockade of LV-TRH prevents hypertrophy and fibrosis in mice. We challenged C57BL/6 adult male mice with an infusion of AngII (osmotic pumps; 2 mg/kg.day) to induce LVH. Groups of mice were injected with an intracardiac siRNA-TRH or scrambled siRNA (siRNA-Con). Body weight, water intake and systolic arterial blood pressure (SABP) were measured daily. AngII significantly increased water intake and SABP ($p<0.05$). Cardiac hypertrophy (heart weight/body weight) was evident in the group with the normal cardiac TRH system. In fact, it was found an AngII-induced increase of TRH precursor mRNA ($p<0.05$) in conjunction with elevated TRH levels measured by immunohistochemistry and western blot. These changes were not observed in the AngII + siRNA-TRH group. Furthermore, AngII increased significantly ($p<0.05$) BNP (hypertrophic marker), collagens I and III and TGF-β (fibrosis markers) expression in the group with the native cardiac TRH system. These increases were attenuated in the groups with the TRH system blocked despite the high blood pressure. Similar and stronger results were observed “in vitro” with NIH3T3 and H9C2 cell culture models, where, when the TRH system is blocked, AngII stimulus was not able to induce the markers of its fibrotic and

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hypertrophic effects, so we believe that these effects are independent of any other physiological modifications.

Our results point out that cardiac TRH is required for AngII-induced hypertrophic and fibrotic effects.

Keywords: Cardiac hypertrophy, TRH, thyroliberin, Angiotensin II, Mice, H9C2, NHI3T3, siRNA.

Non standard abbreviations: AngII angiotensin II, LV left ventricle, LVH left ventricular hypertrophy, siRNA small interference RNA.

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1. Introduction

The renin–angiotensin system has been strongly implicated in hypertension and its complications, and, it has been suggested that the mechanisms by which angiotensin II (AngII) raise blood pressure may be different from those involved in the cardiovascular remodeling and end-organ damage [1]. Indeed, many downstream signaling cascades and target genes/proteins of Ang II have been identified, although cardiac effects such as hypertrophy and fibrosis, independent of blood pressure, remains largely unclear [2,3].

Based on the fact that more than 70% of the Thyrotropin Releasing Hormone (TRH) is produced in extra hypothalamic areas and had been found in cardiac tissue, we have investigated and reported that the spontaneously hypertensive adult rat (SHR) shows left ventricular (LV) TRH hyperactivity, and its long-term inhibition prevents fibrosis and cardiomyocyte enlargement [4]. These results suggested that the cardiac TRH system participates in the development of hypertrophy. In addition, LV overexpression of TRH induces some features of the hypertrophied heart in healthy Wistar rat [5], showing an active role of TRH in cardiac hypertrophy.

Data from other laboratory indicated that the primary source of preproTRH in the heart were fibroblasts [6], and less expression was observed in cardiomyocytes. We confirmed these results and described that cardiomyocytes and fibroblasts stimulated with exogenous TRH induced hypertrophic and fibrotic markers [5]. Moreover, the specific type 1 TRH receptor was also found in the heart [7] and

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belongs to the G protein family [8], which includes the AngII receptor type 1 (Agtr1) and the endothelin receptor type a, both inducers of hypertrophy and fibroblast proliferation.

In addition, Jankowski et al proposed a hypothetical mechanism of TRH-mediated hypertrophy by which the stimulation of cardiac TRH receptors enhances fibroblast proliferation and secretion, and simultaneously cardiomyocyte hypertrophy [9]. Since then, the question of whether cardiac TRH is a common mediator of both fibrosis and hypertrophy has not been elucidated. On this line of thought we propose that LV hypertrophy and fibrosis induced by AngII is mediated by the cardiac TRH system and we speculate that the specific LV TRH inhibition will attenuate fibrosis and/or hypertrophy induced by AngII in C57 mice, despite the high blood pressure.

Therefore, the aim of the present study was to demonstrate that AngII-induced cardiac effects, including fibrosis and hypertrophy are mediated by LV TRH and if its cardiac inhibition attenuates heart damage.

2. Methods

2.1 Ethics Statement

The Institutional Animal Care and Use Committee approved the animal experimentation protocols following the Ethical Guidelines. The protocol was approved by the CICUAL Instituto de investigaciones Médicas (IDIM) UBA-CONICET, Buenos Aires University (UBA) Argentina (Res Nº 2331; November 8, 2011). All manoeuvres were performed under anaesthesia i.p. (unique dose)

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ketamine (90 mg/kg) and xylasine (10 mg/kg), and all efforts were made to minimize animal suffering following the NHI Ethical Guidelines.

C57BL/6 male mice were housed in a room with controlled temperature (23±1°C) under a 12-hour light/dark schedule.

2.2 In vivo treatment

We performed two consecutive protocols exposing mice to a 7-day and 14-day continuous infusion of AngII (2 mg kg^{-1} d^{-1}), a concentration known to establish chronic hypertension and cardiac hypertrophy [10, 11]. In addition, subgroups of AngII-treated animals were assigned in a random blind fashion to one of the two subgroups as follows: specific TRH-siRNA or scramble Con-siRNA (n=8 per group), and were anesthetized (ketamine, 90 mg/kg; xylasine, 10 mg/kg). SiRNA intra-cardiac injections were conducted simultaneously with the osmotic pump (ALZET®) implanted dorsally to maintain the knockdown effect during all the experiment as previously described [4]. All injections were administered under echography. A unique injection of fifty ul of siRNA (10 µg) or saline was injected in the left ventricle (LV) wall. The probe was aligned with the needle and special care was taken to see the region of the myocardial wall. SiRNA oligonucleotides were dissolved in saline. One injection was enough to maintain the knockdown effect during the experiment (14 days). All experiments were performed blindly with respect to the treatment.

Body weight, systolic arterial blood pressure (SABP) and water intake were recorded during the experiments.

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2.3 SABP measurement:

SABP was measured by determining the tail blood volume with a volume pressure recording sensor and an occlusion tail-cuff (CODA; Kent Scientific, Torrington, CT). Prior to this, the animals were put in a quiet room for 30 min before the measurement of SABP and heart rate. Each value corresponds to at least three independent measurements.

2.4 Tissue samples:

Animals were euthanized by decapitation; the hearts were rapidly removed and processed according to the protocol for peptide and mRNA determinations. Blood samples were collected for thyroid hormone and TSH measurements.

2.5 Gene expression quantifications:

Total RNA was extracted from cardiac tissue. Gene expression quantifications were performed using a real-time RT-PCR technique normalized by a housekeeping gene (β-actin) expression. Briefly, for cDNA synthesis, 1 μg of total RNA and 1 μg of random hexamer primers (Promega Corp, WI, USA) were heated 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 $MgCl_2$, 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 37ºC for 1 h, followed by inactivation of the enzyme at

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95ºC 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 three-step protocol (95°C for 30 sec, 62°C annealing for 30 sec, 72°C for 40 sec) was repeated for 40 cycles.

A melt curve analysis was performed after every run to ensure a single amplified product for every reaction. The size of the amplicons generated was confirmed on a 2% agarose gel. In accordance with the literature, we did not find any differences between experimental groups in the β-actin housekeeping gene by the GeNorm software. Quantification was performed by normalizing Ct (cycle threshold) values with β-Act Ct and analyzed with the $2^{-\Delta\Delta}$CT method.

Mouse primers were from Invitrogen, (Invitrogen, CA, USA), listed in supplementary file.

2.6 Pathology examination

Mice hearts were perfuse with saline solution through the abdominal aorta until they were free of blood. Tissue was fixed in phosphate-buffered saline (PBS) 10% formaldehyde (pH 7.2) and embedded in paraffin. Three-micron sections were cut and stained with haematoxylin-eosin (H&E), Masson's trichrome and Sirius red. The image analysis technique was carried out on ten microscopic fields at a magnification of 400x scanned in the form of Greek guard. All observations in light microscopy were performed using a Nikon E400 light microscope (Nikon

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Instrument Group, Melville, N.Y., USA), and the observer was blind to the animal group.

2.7 Immunohistochemical evaluation

Immunolabelling 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 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 biotinylated universal antibody for 30 min. After a final wash in PBS, 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 TRH content by immunohistochemestry, the specific anti-rat/mouse TRH polyclonal antibody was used at a dilution of 1:100 (sc-366754, Santa Cruz, Inc., USA), for TGF-β1 a polyclonal antibody was used at a dilution of 1:300 (sc-146, Santa Cruz, Inc., USA) and for collagen type I a rabbit polyclonal antibody was used at a dilution 1:100 (ab34710, Abcam, Cambridge, MA). For the evaluation of alpha-actin content, the alpha-cardiac actin (Alpha Sr-1): sc-58671 Santa Cruz Biotech mouse monoclonal antibody was used at a dilution 1:100 (images 600x) and for myosin evaluation

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the myosin heavy chain (MYH7) (A4.951) sc-53090 Santa Cruz Biotech mouse monoclonal antibody was used at a dilution 1:100 (images 600x).

2.8 Cell culture

Rat cardiomyoblasts (H9c2) (ATCC) were seeded (1×10^4 cells/cm^2) and cultured in Dulbecco's modified Eagle's medium (DMEM-F12) supplemented with 10% fetal bovine serum (FBS), 50 µg/mL penicillin, and 50 µg/mL streptomycin and Fungizone (GIBCO) (250 µg/mL) under a normal culture condition (5% CO_2, 37°C). Twenty-four hours before treatment, we switched to the differentiation medium (DMEM-F12; 1% FBS and 50 nM trans-retinoic acid RA, R2625; Sigma).
Mouse fibroblasts (NIH3T3) were obtained from the ATCC and cultured in DMEM-F12 supplemented with 5% FBS, 50 µg/mL penicillin, and 50 µg/mL streptomycin and Fungizone (GIBCO) (250 µg/mL) under a normal culture condition (5% CO_2, 37°C). Twenty-four hours before treatment, cells were switched to DMEM-F12 without FBS.
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 siRNA concentration (ul/ug) were selected to get the best result (4/1).

2.9 Immunofluorescence

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Cells was fixed with 4% formaldehyde for 20 min, washed with PBS (2 times), blocked with 3% FBS for 30 min at room temperature, and then incubated with primary antibodies, anti-TRH (SC-366754, 1/500) or anti-TGF-β1 (SC-146, 1/300) (Santa Cruz, Inc., USA) overnight at 4°C. After washing with PBS, they were incubated with the secondary antibody, Alexa Fluor 568 anti-rabbit (A10042) and Alexa Fluor 546 anti-mouse (A10036) (Invitrogen, Inc., USA), for 1 h at room temperature. Those samples were rinsed again with PBS and mounted in a mounting medium, with DNA-specific 4′,6-diamidino-2-phenylindole (DAPI; H1200; Vector Lab.) treatment. Fluorescence was observed using a confocal laser scanning microscope.

2.10 Morphological and quantitative analysis

All tissue samples were evaluated independently by two investigators without knowing the group to which the sample belonged. All measurements were carried out using an image analyser, Image-Pro Plus version 4.5 for Windows (Media Cybernetics, LP, Silver Spring, Maryland, USA).

2.11 Biochemical and pathology studies

At the end of the treatment, mice were weighed, and the snout-tail long index was measured before the animals were sacrificed by decapitation. Blood samples were collected with sodium EDTA, and thyroid hormone levels were measured using an enzyme immunometric assay (EIA) (Assay Designs, Inc., USA). Hearts were rapidly removed and weighed. In some animals, cardiac tissues were separated

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(atria, left ventricle, right ventricle, and septum) for protein and mRNA quantification; others were used for pathology studies.

2.12 Western Blot

Left ventricular tissue was homogenized in RIPA lysis buffer (50 m M Tris-HCl, pH 7.4, 150 mM NaCl, NP-40 1%, SDS 0,1% (W/V), Sodium Deoxicholate 0.5% (W/V)) with protease inhibitors (2ug/ml aprotinin, 7,5 μg/ml leupeptin, PMSF 1mM and EDTA 5mM). Total protein concentration was measured using Bradford assay. Subsequently, 40 μg of total protein from each sample was added to loading buffer (4% SDS, 10% 2-mercaptoethanol, 20% glicerol, 0,004% bromophenol blue, 0,125 M Tris-HCl), and was resolved by SDS-12% PAGE and electro blotted onto PVDF membranes (Thermo Scientific, Rockford, USA). After blocking for 1 h in TBST containing 5% non-fat milk, membranes were probed with a rabbit anti TRH precursor (Abcam, Cambridge, MA, USA, 1:1000, ab171958) or a TGF-β1 antibody (Santa Cruz Biotechnology Inc., USA, 1:100, sc-146) at 4 °C overnight and were washed three times in TBST. Then, membranes were incubated with goat anti-rabbit IgG peroxidase conjugated antibody (Santa Cruz Biotechnology, 1:3000 sc-2004) and bands were visualized by chemiluminescence (Clarity ™ ECL Substrate, Bio-RAD Laboratories, Florida, USA) according to the manufacturer's instructions and exposured to x-ray films (Carestream Healt inc, Rochester, NY, USA). The housekeeping gene β-Actin (Abcam, Cambridge, MA, USA 1:1500, ab6276) was used as load control. For quantification, we used Image-Studio Lite

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software 5.2 version (Li-cor Biotechnology, Lincoln, NE, USA). The relative level of protein expression was expressed as the ratio to β-Actin.

2.12 Statistics

Values are expressed as mean ± standard error of the mean (SEM). All statistical analyses were performed using absolute values and processed using Statistics, version 6.0 (Software, Inc., California, USA). The assumption test to determine Gaussian distribution was performed using the Kolmogorov-Smirnov method. For parameters with Gaussian distribution, comparisons between groups were carried out using one-way analysis of variance (ANOVA) followed by Fisher test. 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.

3. Results

3.1 Efficacy of the Ang II infusion on blood pressure, daily water intake and body weight.

As expected, Ang II infusion was able to increase blood pressure (panel A) and daily water intake (panel B) as its values were significantly higher in the Ang II groups evidencing the dipsogenic and hypertensive effect of the octapeptide, in an independent manner to the siRNA treatment. As shown in panel c, body weight was higher in all the treated groups and shows the expected increase after two weeks with respect to the basal group, without differences in the Ang II groups.

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3.2 One week of AngII infusion induces a slight increase of left ventricle TRH precursor without changes in hypertrophic index.

After one week, as shown in figure 2 panel A, we observed a slight left ventricle TRH increase that was accompanied by minor increases in fibrotic and hypertrophic markers gene expressions (panels B and C). When we analyzed the hypertrophic index between the groups, we unexpectedly did not find any changes between groups. This shows that although modest molecular alterations were observed, macroscopic damage was not evident. Assuming that this result could be due to the short duration of the treatment, we decided to challenge the animals with a longer AngII infusion (up to 14 days).

3.3 Long-term AngII infusion requires an intact left ventricle TRH system to induce LVH.

In line with our assumption, as shown in figure 3 panel A, we found a significant increase in the hypertrophic index that was only evident in the group treated with AngII with an active TRH system. Conversely, this increase was not seen in the group infused with AngII but with the TRH system blocked, which presented an index similar to the control group even in the presence of high SABP. Moreover, when we evaluated cardiomyocytes diameter in these animals we found a significant increase in Ang II group with a native TRH system and as expected this cardiomyocyte diameter increase was significantly attenuated in the group receiving Ang II with the TRH system blocked.

Figure 3, panel B shows that the 14-days AngII infusion induced a more than 10-fold increase in the preTRH gene expression (AngII+siRNA-CON), evident only in

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the subgroup with a native TRH system compared with the control and AII+siRNA-TRH groups, demonstrating the tripeptide induction by AngII and the efficacy of the long-lasting effect of the TRH inhibition manoeuvre. We confirmed this result by the measurement of TRH precursor by western blot where we found that the increase of TRH induced by Ang II was completely reversed in the group in which the TRH system was inhibit.

In association with higher TRH expression, the long-term AngII infusion induced significant increases in both hypertrophic, (β-MHC and BNP) (panel C), and fibrotic, (TGF-β1 and collagen types III and I), recognized markers being stronger in the last ones (Figure 3 panel D and E respectively). These increases were seen in the subgroup with a functional TRH system (AII+siRNA-Con). On the contrary, hypertrophy and fibrosis markers gene expressions were highly attenuated when the TRH system was inhibited, as were similar to those in control groups. As changes in gene expression might not reflect protein status, we first performed western blot and found TGF- β1 induction by Ang II (around 100%); this increase was significantly reduced in the siRNA TRH treated group. We also performed immunohistochemistry for both peptides TRH precursor and TGF- β1 as shown in Figure 4 (panel A). AngII infusion markedly induced TRH precursor expression, showing a brown immunohistochemical staining in the AngII group with a functional TRH system that was reduced to the 50% in the group with the TRH system blocked. TRH stain was not significant in the control group (left). A similar pattern was found for TGF-β1 (right). In fact, AngII-induction of TGF-β1 protein was 3 folds- higher in the group with a functional TRH system, and was significantly decreased in the group with a blocked TRH system, although did not reach control

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values. As shown in Figure 4 (panel B), we observed the stimulation of collagens in the extracellular matrix (Sirius Red , left) that correlates with the significant increase observed in collagen type I (right) by AngII, maximal in the group with AngII with an active TRH system. Moreover we observed approximately a 50% decrease in both signals in the AngII-treated group with the TRH system inhibited. The evaluation of Masson's Trichrome staining showed a similar pattern (supplemental section) pointing out that the TRH system is necessary for long-term AngII full fibrotic effects.

We evaluated two proteins strongly related with hypertrophy development, particularly α-cardiac actin and myosin heavy chain. As shown in figure 4 panel C we found that AngII treatment induced both protein, the inhibition of the TRH system significantly attenuated these increases, although did not reverse them completely; this confirm the data obtained in cardiomyocyte diameter.

3.4 Effect of the TRH inhibition on the fibrosis induced by AngII in the NIH3T3 fibroblasts cell line.

Considering that the heart is composed of several cell types, we decided to evaluate the effect of TRH inhibition on AngII treatment in two cell lines, mouse fibroblasts (NIH3T3) and rat cardiomyocytes (H9C2). Both cell lines have been studied for many years as models of cardiac cells and have been well characterized and validated [12].

First, we studied NIH-3T3 fibroblasts and, we confirmed the TRH induction by AngII (1uM) by immunofluorescence showing a strong sparkling signal in the cells stimulated by AngII with the native TRH system intact in contrast with the cells

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incubated with saline, which presents a very low signal (Figure 5, panel A). The AngII stimulus induces not only a significant increase in TRH peptide content but also markedly increases of the TGF-β1 and collagen types I and III gene expressions (panel B). To answer the question as to whether the increase of all the fibrotic markers, TGF-β1 and collagens, may be a consequence of TRH induction induced by the AngII stimulus, we evaluated the effect of AngII along with TRH inhibition over these markers. In agreement with *in vivo* experiments, siRNA-TRH treatment was effective in inhibiting TRH expression. AngII stimulus only increased TRH in the group carrying the native TRH system and no increase was observed in the fibroblasts stimulated with AngII with the inhibited TRH system, in which TRH expression was similar to control cells. Thus, when the TRH system is blocked, AngII stimulus was not able to induce its fibrotic effect, and neither did TGF-β1 nor collagens type I and III change their expression 24 hours after AngII stimulus (Figure 5, panel C). Finally, we confirmed that the AngII induction over TRH expression was mostly mediated by its specific receptor, Agtr1, being that the losartan (LST) antagonist before to AngII hampers the TRH induction (Figure 5, panel D).

3.5 Effect of the TRH inhibition on the hypertrophy induced by AngII in H9C2 cardiomyocytes cell line.

Secondly, we used cultured cardiomyocytes (H9C2 cells) and confirmed that AngII increased the TRH (peptide) content by immunofluorescence, showing a strong sparkling red signal in the cells stimulated by AngII with the native TRH system intact in contrast with the cells incubated with saline, which presents a very low

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signal. Thereby, Figure 6, panel B shows a 2-fold increase of the TRH precursor mRNA and peptide only in the group stimulated with AngII with an intact TRH system compared to the culture cells with the specific TRH inhibition by siRNA-TRH, in which TRH peptide levels were not different from that of control cells. As expected, TRH increase induce hypertrophic markers expression as seen in Figure 6 panel C, where the increases of TGF-β1 and BNP were only evident after AngII stimulus in the cells where the TRH system was active. Furthermore, the group stimulated with AngII with the TRH system suppressed was similar to the cells receiving saline with or without native TRH system active. A similar pattern was observed with the measurement of α-actin expression, a sarcomeric protein strongly related to hypertrophy, which was increased by AngII stimulus only in the group carrying a working TRH system.

4. Discussion

In this study, we propose the cardiac TRH as a common mediator in the development of the LVH, in particular in the AngII-induced hypertrophy. This work shows that AngII stimulates cardiac TRH and that TRH inhibition attenuates fibrotic and hypertrophic effects induced by AngII, thus making the cardiac TRH system a promising therapeutic target.

Therefore, we treated C57BL/6 mice with saline, as a control and AngII to induce cardiac hypertrophy with and without a specific siRNA inhibition of the TRH system. We found that AngII definitely stimulates cardiac TRH. This stimulus was shown to be time-dependent, as we observed that the TRH increase after 7 days of AngII infusion was significantly lower than the one observed after 14 days of Ang II

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infusion. As a result, cardiac TRH exerted pro-hypertrophic and pro-fibrotic cardiac effects. It is remarkable that in the short protocol where the TRH increase was mild and close to 50% above to that observed in controls, the increases in hypertrophy and fibrosis markers were also mild. Accordingly, we did not find macroscopic hypertrophy as the hypertrophic index was not different compared to controls, revealing that these gene alterations were just the beginning of the process. However, after 14 days of AngII infusion, there was a significant TRH increase confirmed by western blot and immunohystochemistry that brought about marked increases in hypertrophic and fibrotic markers. In this scenario, we found a significant increase in the hypertrophy index, indicating a well established hypertrophy induced by AngII only in the group with a functional TRH system. These findings reinforce the concept of an important role of TRH in LVH development.

We found that increases in TRH gene expression were not reflected in precursor protein levels, probably due to a rapid processing of the preTRH to the tri-peptide which brings out the observed effects. Indeed we found an increase of more than 3 fold in the expression of PC1 convertase, which process pro-TRH to cryptic peptides (data not shown); although, we cannot dismiss that the TRH precursor is secreted from the cell. Western blot results (TRH precursor and TGF-β1) were as not strong as immunohystochemistry results although, were confirmatory of the effect directions. Probably this quantitative discrepancy could be due to differences in the antibody used for each technique. Nevertheless, assuming that TRH inhibition was not completely achieved, it can be speculate that with a stronger inhibition, the effects observed could be totally prevented.

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As a whole, our results indicate that there is a positive correlation between LV TRH and hypertrophy/fibrotic markers, in which exposure time to the insult (in this study AngII) is a central issue.

Our results highlight the relevance of the cardiac TRH system under pathological situations as no changes have been observed between animals receiving saline with an active or suppressed TRH gene expression.

Decreases in TRH by 50%-70% were efficient to attenuate strongly fibrosis and also hypertrophy which suggest a threshold over which TRH participates actively in the development of this pathology.

Furthermore, we showed that cardiac TRH is located downstream to AngII in the LVH development and point out the relevance of the TRH system in situations where the renin–angiotensin system is enhanced. It has been described by Bacova et al [13] that the transgenic rat model (mRen2) with an extra copy of the mouse renin 2 gene, which induces higher Ang II levels in both plasma and heart, increased the preTRH gene expression in the left ventricle. Related to our work, preTRH gene expression has been shown to be induced in the left ventricle of rats after myocardial infarction [14] where, AngII is overexpressed suggesting that maneuvers directed to modulate the cardiac AngII system would probably affect the TRH system in the heart. Indeed, TRH can increase cardiac performance in rats with ischaemic cardiomyopathy [6].

On the contrary, Jin et al described that an early treatment with ACE inhibitors normalized many genes found to be increased in heart failure [6], but unexpectedly, the expression of preTRH in left ventricle is increased. Whether this increase represents compensatory mechanism with detrimental effects remains to

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be studied, even so we speculate that the inhibiting of TRH may be a useful therapeutic approach.

The TRH induction evoked by AngII is evident in cardiac fibroblasts and cardiomyocytes, the two major cell components of the heart. Even more, both types of cells express the Ang II type 1 receptor [13], in addition to the specific TRH type I receptor and all the machinery necessary to express TRH as was reported previously [14,6]. Indeed, we have described previously that TRH overexpression was able to induce hypertrophic and fibrotic markers in rat primary cell culture of both myocytes and fibroblasts [5]. In line with those results, using validated models of myocytes (H9C2) and fibroblasts (NIH 3T3) we were able to show that AngII induce an increase of TRH and a significant increase of hypertrophic markers, (BNP, β-MHC and TGF-β1), and expansion of ECM in both cell types. Furthermore, when TRH system is disrupted, the AngII effects were not observed, pointing out that TRH is an important mediator of AngII action in both cell types. We observed that although the tripeptide increase by immunofluorescence was similar in both type of cells, RNA expression was higher in fibroblasts probably due to differences in translation, traduction or processing. In myocytes, we found a mild 2-fold TRH increase compared to control, which was able to induce changes of the same magnitude on the hypertrophic markers, reinforcing the concept that higher levels of TRH implies higher damage to cardiac tissue.

It is known that neonate cells could respond in a different way compared to adult cardiac cells, and we know the limitations on working on a cell line. Nevertheless, cell culture results confirmed an Ang II-induced TRH action on cardiac cells.

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Finally, we have tested “in vitro” our hypothesis that the TRH system is essential for the cardiac remodeling induced by AngII independently of any cardiovascular effect.

It has to be mentioned that using rat heart slices Ang II significantly inhibited the secretion of TRH [13], which may explain partially our results as in this study we assessed TRH cell content. We think that “in vitro” results strongly support the “in vivo” observations and point out that cardiac TRH mediates AngII cardiac effects. It is tempting to postulate the heart TRH system as a therapeutic target in cardiac hypertrophy. In fact, our siRNA-TRH performed well as a long-lasting inhibitory agent.

Further investigations will be needed to elucidate the transduction signal pathways activated by TRH in heart tissue, so cardiac TRH is a novel mediator, perhaps a crucial step, in the cardiac effects induced by AngII and possibly other insults.

4.1 Perspectives

Our results put in evidence the role of the cardiac TRH system in heart pathophysiology especially in the disorders that involve the induction of AngII given that cardiac TRH acts like an AngII mediator.

We have shown a siRNA designed against TRH which seems to be an effective strategy to knockdown the TRH system of the left ventricle that hinders the development of fibrosis and hypertrophy induced by AngII, opening the possibility of the management of the TRH system in situations in which the system is overexpressed.

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7. Conflict of interest: none declared.

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Figure Legends:

Figure 1: Ang II treatment on systolic arterial blood pressure, daily water intake and body weight.

Effects of the Ang II treatment (2mg/kg/day) in mice on systolic arterial blood pressure (SABP) (A), daily water intake (B) and body weight (C) compared with basal period (n=8/group). C57 adult mice were intracardiacally injected with siRNA-Con or siRNA-TRH previously to AngII infusion compared with their basal. Results are expressed as media±SE. ANOVA $^*p<0.05$, compared to basal group (n=8).

Figure 2: Effect of 7-days Ang II infusion on left ventricular TRH and hypertrophic and fibrotic markers expression.

Panel A shows left ventricle TRH precursor (pre-proTRH). In panel B and C, gene expression of type III collagen and βMHC and brain natriuretic peptide (BNP) are shown as fibrotic and hypertrophic markers. Gene expression was determined by real-time PCR and normalized by ß-actin expression. C57 adult mice that were intracardiacally injected with siRNA-Con or siRNA-TRH previously to 7 days AngII infusion (2 mg/kg/day) compared with their control receiving saline (n=12/group). Results are expressed as percentage of control group. ANOVA $^*p<0.05$, compared with saline group.

Figure 3: Long-term Ang II infusion requires an intact left ventricle TRH system to induces LVH

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Hypertrophy index and cardiomyocytes diameter (panel A), left ventricle TRH precursor, mRNA expression and peptide, (panel B) ; βMHC and BNP genes expression (panel C), TGF-β1 gene expression and protein (panel D) and types III and I collagen gene expression (panel E) of C57 adult mice that were intracardiacly injected with siRNA-Con or siRNA-TRH previously to a long 14-day AngII infusion (2 mg/kg/day) compared with their control receiving saline (n=12/group). Genes expression was determined by real-time PCR normalized by ß-actin. Quantization of the TRH precursor and TGF- β1 protein was done by western blot. Results are expressed as percentage of control group. ANOVA *p<0.05, compared with saline group.

Figure 4: The long-term AngII infusion induces increases of TGF-β1, collagen type I and expansion of the ECM, all avoided by the left ventricular TRH inhibition.

Effect of left ventricle TRH inhibition on ECM expansion in mice treated with 14 days AngII infusion (2mg/kg/day). TRH and TGF-β proteins expression (panel A) and ECM expansion by Siriud Red staining and collagen type I (panel B). Note significant difference in ECM expansion (arrows) between groups (Masson's Trichrome). Arrows indicate areas with different degree of fibrosis (left) and positive immunostaining for collagen type I (right) in both AngII-treated groups, (n=5). Tables represent the quantification of TRH and TGF-β proteins and ECM expansion areas in all groups. Results are expressed as mean ± SD. ANOVA *p<0.05, compared with saline group; #p<0.05, versus AngII + siRNA-TRH.

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Figure 5: An AngII stimulus induces the increase of TRH which act as a mediator of its fibrotic effects in fibroblasts NIH-3T3.

Twenty-four hours AngII (1uM) induction on TRH protein content and TGF-β and collagen type I genes expressions, determined by immunofluorescence and real-time PCR normalized by ß-actin expression, respectively, in NIH-3T3 cell culture (panel A), n=7.

Effect of the TRH inhibition (siRNA transfection), on AngII induction (1 μM, 24 h) over fibrotic markers in NIH-373 cell line. TRH-precursor, TGF-β and collagen type I genes expression determined by real-time PCR normalized by ß-actin expression (panel B). Effect of losartan, (LST, 10 μM 30 min. before stimulus), on the induction of TRH and TGF-β genes expression by AngII (1uM). Results are expressed as percentage of control group. ANOVA $*p<0.05$, compared with control group, n=7.

Figure 6: TRH mediates the hypertrophic effect of the AngII in H9C2 myocyte cell line.

Effect of TRH inhibition (siRNA transfection), on AngII induction (1μM, 24 h) over hypertrophic markers in myocytes H9C2 cell line. TRH precursor RNA, TRH peptide (panel A), TGF-β and BNP (panel B) genes expression determined by immunofluorescence and real-time PCR normalized by ß-actin expression, respectively. Results are expressed as percentage of control group. ANOVA $*p<0.05$, compared with control group, n=7.

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Highlights

- TRH is a crucial step in the cardiac effects induced by AngII
- In NH3T3 and H9C2 the AngII requires TRH to induce fibrotic and hypertrophic markers.
- TRH-siRNA is an effective strategy to impede fibrosis/ hypertrophy induced by AngII

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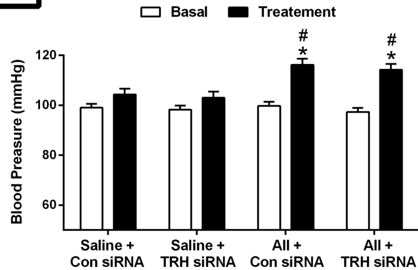
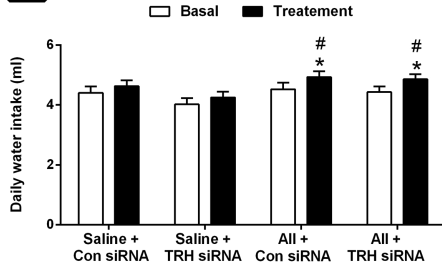




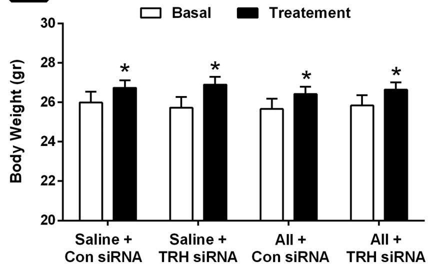
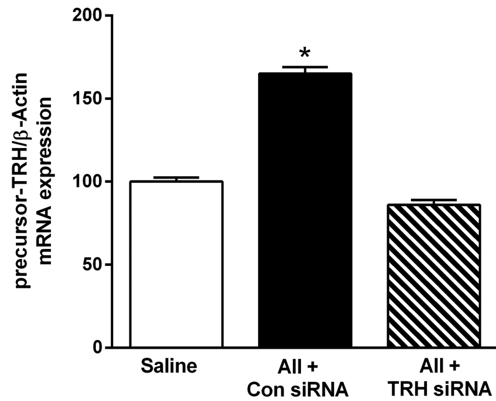
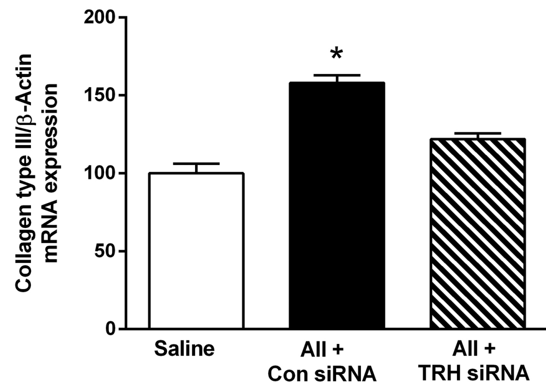
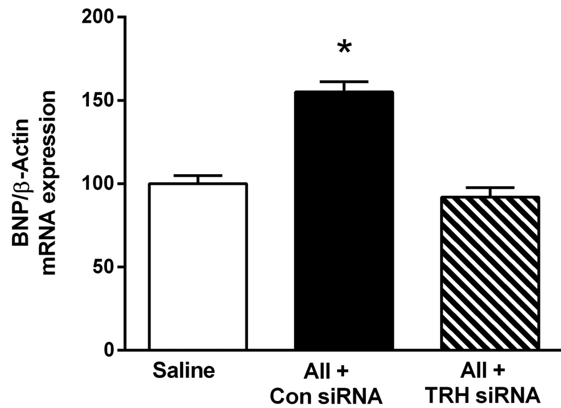


Figure 1







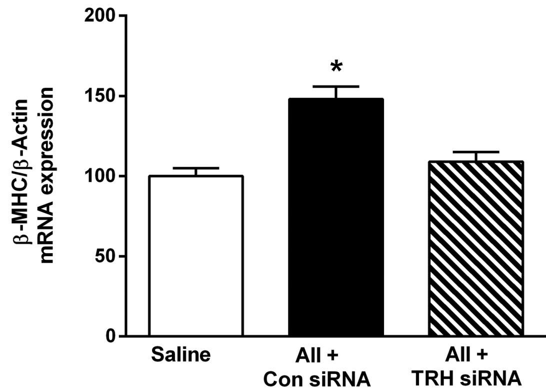


Figure 2

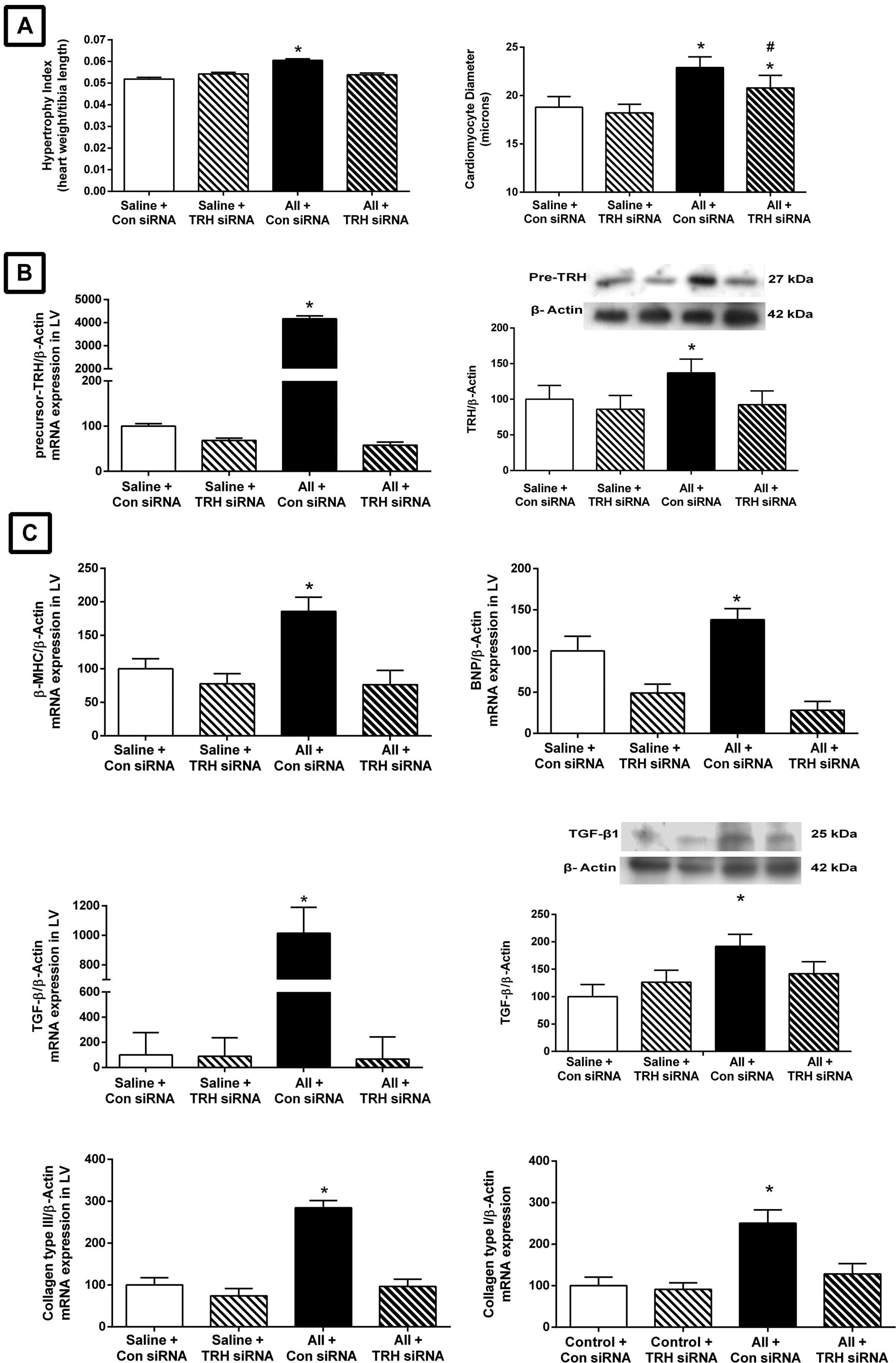
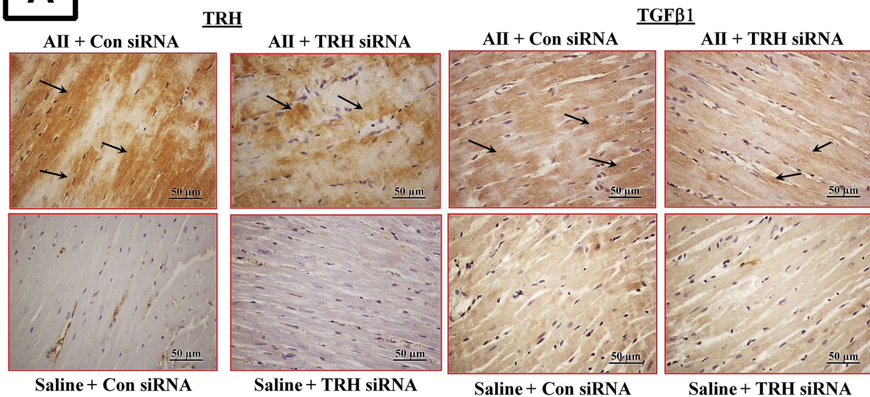
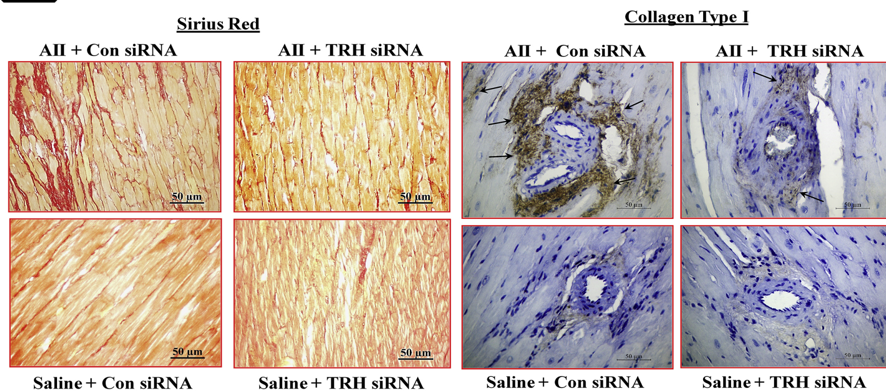


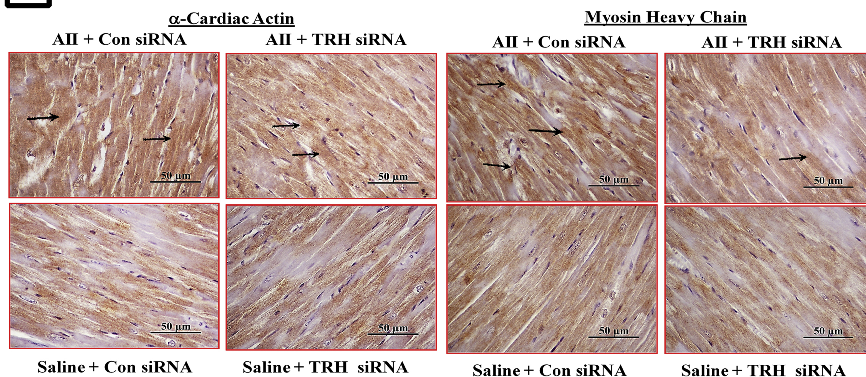
Figure 3



Mean ± SD	AII + Con siRNA	AII + TRH siRNA	Saline + Con siRNA	Saline + TRH siRNA
TRH (left) (Positive staining (%) / area)	24.9 ± 2.5*	18.7 ± 1.5*#	2.6 ± 0.7	2.1 ± 0.6
TGF-β1 (right) (Positive staining (%) / area)	30.2 ± 2.4*	10.7 ± 2.5*#	5.8 ± 1.0	5.3 ± 1.0



Mean ± SD	AII + siRNA-Con	AII + siRNA-TRH	Saline + siRNA-Con	Saline + siRNA-TRH
Sirius Red (Positive staining (%) / area)	17.3 ± 1.9*	9.6 ± 1.5*#	2.5 ± 0.9	2.0 ± 0.8
Collagen Type I (right) (Positive staining (%) / area)	11.3 ± 2.7*	6.4 ± 1.3*#	2.4 ± 0.5	1.7 ± 0.3



Mean ± SD	AII + Con siRNA	AII + TRH siRNA	Saline + Con siRNA	Saline + TRH siRNA
α-Cardiac Actin (left) (Positive staining (%) / area)	51.5 ± 3.0*	45.0 ± 2.3*#	39.7 ± 1.4	38.1 ± 2.1
Myosin Heavy Chain (right) (Positive staining (%) / area)	48.2 ± 3.4*	41.8 ± 2.4*#	5.8 ± 1.0	5.3 ± 1.0

Figure 4

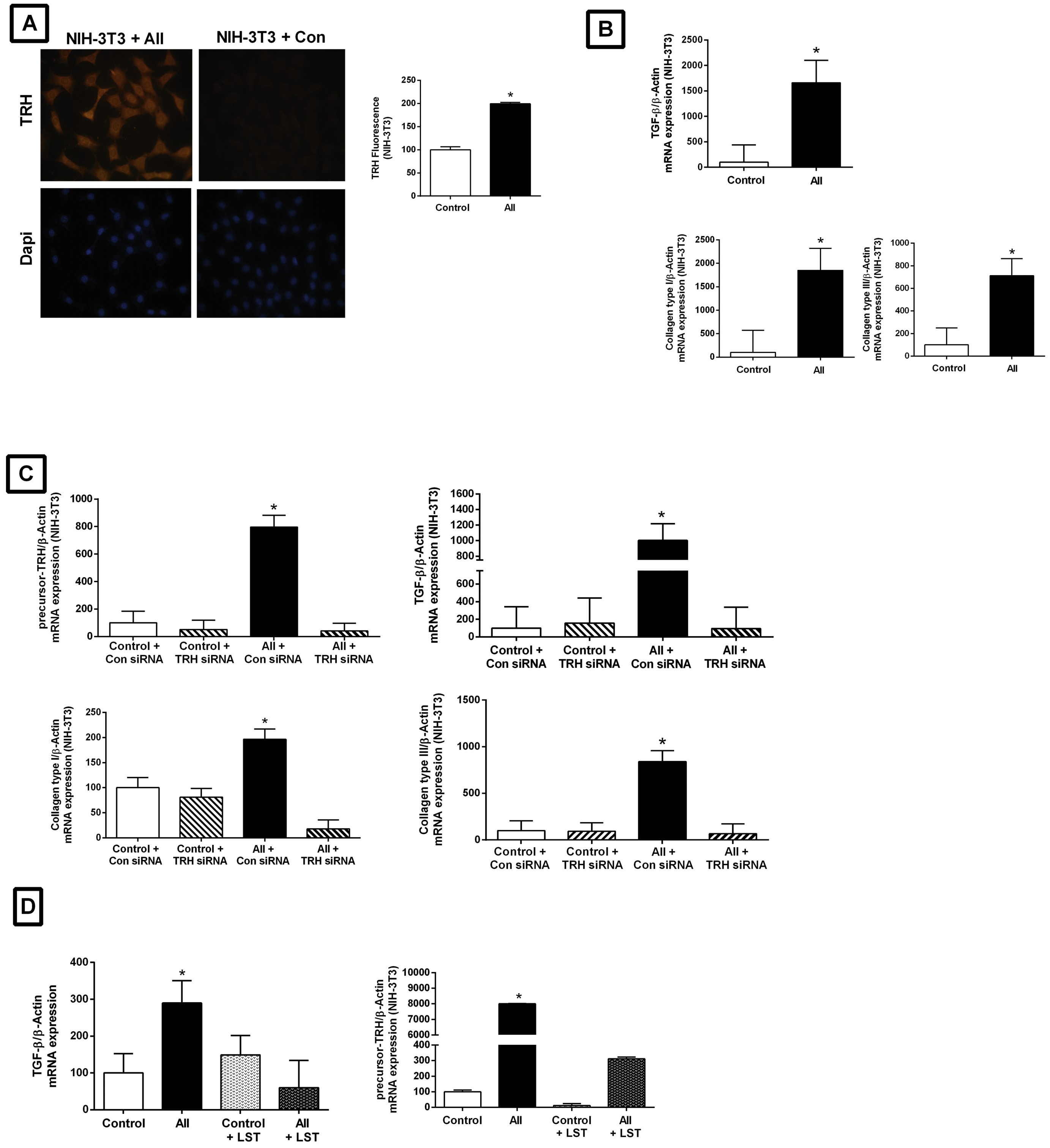
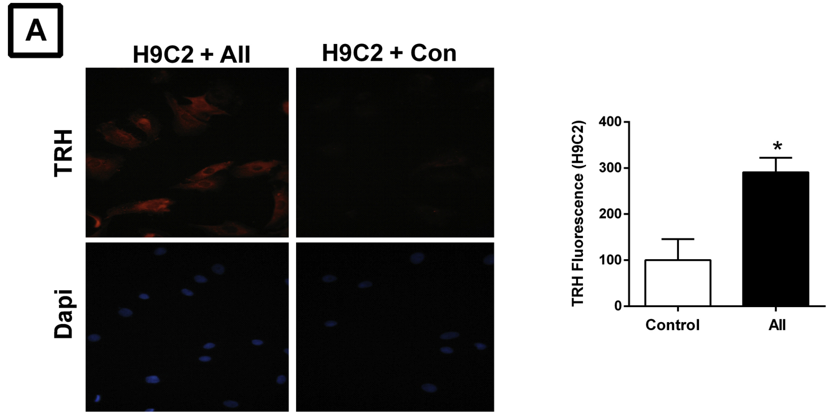
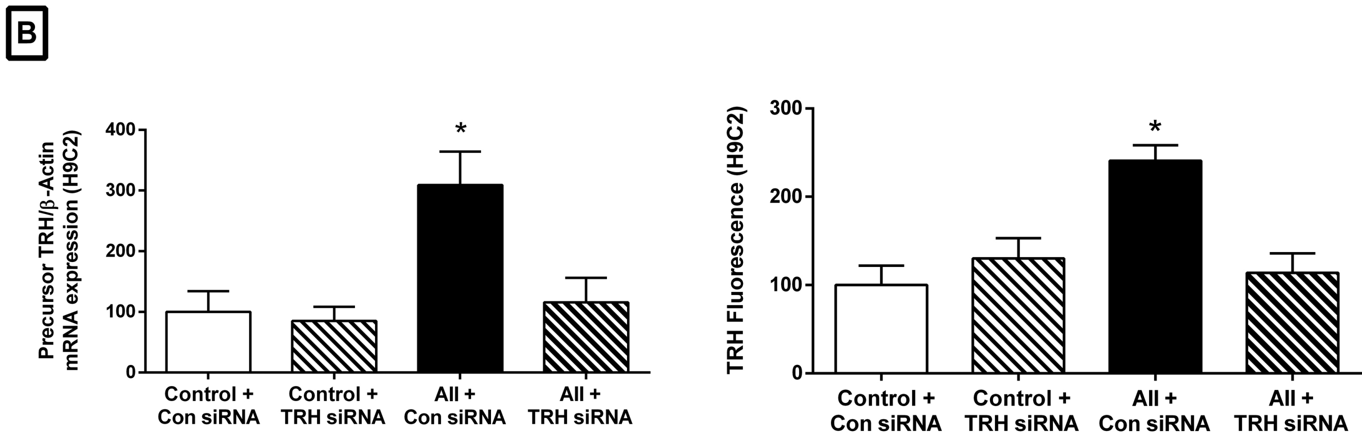


Figure 5





C

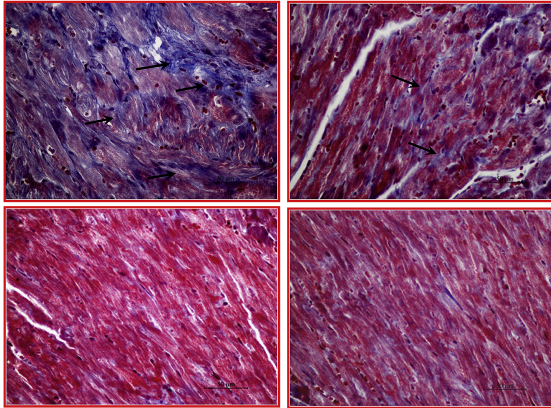
TGF-β/β-Actin mRNA expression (H9C2)
600
400
200
0
*
Control + Con siRNA
Control + TRH siRNA
AII + Con siRNA
AII + TRH siRNA

BNP/β-Actin mRNA expression (H9C2)
300
200
100
0
*
Control + Con siRNA
Control + TRH siRNA
AII + Con siRNA
AII + TRH siRNA

α-Actin Fluorescence (H9C2)
200
150
100
50
0
*
p<0.001
Control + Con siRNA
Control + TRH siRNA
AII + Con siRNA
AII + TRH siRNA

Figure 6

A



Mean ± SD	AII + Con siRNA	AII + TRH siRNA	Saline + Con siRNA	Saline + TRH siRNA
Masson's Trichrome (left) (Positive staining (%) / area)	22.3 ± 2.2*	12.1 ± 2.5*#	3.4 ± 0.9	3.7 ± 1.1

Effect of left ventricle TRH inhibition on ECM expansion in mice treated with 14 days AngII infusion (2mg/kg/day). ECM expansion by Masson's Trichrome staining, note significant difference in ECM expansion (blue). Arrows indicate areas with different degree of fibrosis, (n=5). Table represent the quantification of ECM expansion area in all groups. Results are expressed as mean + SD. ANOVA *p<0.05, compared with saline group; #p<0.05, versus AngII + siRNA-TRH.

Figure 7