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Original Paper

The Impact of a Non-Functional Thyroid Receptor Beta upon Triiodotironine-Induced Cardiac Hypertrophy in Mice

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Key Words
Thyroid Hormone • Cardiac hypertrophy • Thyroid hormone receptor • MicroRNA • Transgenic mice

Abstract
Background/Aims: Thyroid hormone (TH) signalling is critical for heart function. The heart expresses thyroid hormone receptors (THRs); THRα1 and THRβ1. We aimed to investigate the regulation mechanisms of the THRβ isoform, its association with gene expression changes and implications for cardiac function. ***Methods:*** The experiments were performed using adult male mice expressing TRβ$^{\Delta 337T}$, which contains the Δ337T mutation of the human *THRB* gene and impairs ligand binding. Cardiac function and RNA expression were studied after hypo- or hyperthyroidism inductions. T3-induced cardiac hypertrophy was not observed in TRβ$^{\Delta 337T}$ mice, showing the fundamental role of THRβ in cardiac hypertrophy. ***Results:*** We identified a group of independently regulated THRβ genes, which includes *Adrb2*, *Myh7* and *Hcn2* that were normally regulated by T3 in the TRβ$^{\Delta 337T}$ group. However, *Adrb1*, *Myh6* and *Atp2a2* were regulated via THRβ. The TRβ$^{\Delta 337T}$ mice exhibited a contractile deficit, decreased ejection fraction and stroke volume, as assessed by echocardiography. In our model, miR-208a and miR-199a may contribute to THRβ-mediated cardiac hypertrophy, as indicated by the absence of T3-regulated ventricular expression in TRβ$^{\Delta 337T}$ mice. ***Conclusion:*** THRβ has important role in the regulation of specific mRNA and miRNA in T3-induced cardiac hypertrophic growth and in the alteration of heart functions.

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Introduction

Thyroid hormone (TH) signalling is critical for proper heart development and function. Triiodothyronine (T3) and thyroxine (T4) can act directly on cardiomyocytes via genomic and non-genomic mechanisms. Additionally, T3 and T4 exert important indirect effects on cardiovascular functions by acting on the vascular system. After binding to nuclear receptors (thyroid hormone receptors; THRs), T3 modulates gene transcription, a function that occurs at specific sequences of DNA called TH-responsive elements in the promoter regions of T3 target genes [1]. Once bound to T3, THRs undergo conformational changes, favouring the release of corepressor proteins and the recruitment of coactivator molecules for the THR-TRE complex [1], resulting in the increased transcription of many positively regulated T3-responsive cardiac genes. In the absence of T3, THRs repress cardiac genes that are positively regulated by THs [2]. Moreover, T3 can bind to plasma membrane thyroid receptors, such as integrin $\alpha_V\beta_3$ [3, 4], activating quick-start signalling cascades, including phosphatidylinositol 3'-kinase (PI3K) and mitogen-activated protein kinase (MAPK) [5], which can regulate ribosomal biogenesis and protein translation and modulate the activity of membrane ions transporters, among other actions [6]. Additionally, non-genomic TH activation can interact with the genomic effects and contribute towards an overall effect on the heart [7, 8].

One of the most important and classic T3 effects on the heart is the induction of cardiac hypertrophy [9]. T3-induced hypertrophy is accompanied by a compensated cardiac functional response, which is primarily based on increased activities and expressions of proteins, such as sarcoplasmic reticulum calcium-activated ATPase (SERCA2, *Atp2a2*), a protein responsible for calcium re-uptake during diastole and increasing the relaxation rate. T3-induced hypertrophy is also accompanied by an increased expression of α-myosin heavy chain (α-MHC, i.e., the fast myosin having higher ATPase activity, *Myh6*) and a reduced expression of β-MHC (i.e., the slow myosin, *Myh7*). As a characteristic response to a stimulus-inducing adaptive hypertrophy, these effects improve cardiac contractility [10].

Additionally, T3 regulates the expression of specific genes implicated in the control of normal growth and prevents the expression of foetal gene patterns, characteristic of pathological cardiac hypertrophy [11, 12]. This regulation is dependent on the THRs, suggesting their important role in the induction of cardiac hypertrophy by T3 [1, 13]. THRs are encoded by two distinct genes in rodents: *Thra* and *Thrb*. Through alternative splicing, these genes have at least four active isoforms, THRα1, β1, β2 and β3 [1]. These isoforms are expressed in various amounts in different tissues and exert varied functions in each [14-18]. Most of the effects of T3 on the heart are suggested to occur via THRα1 [19-21], which is the predominant receptor isoform expressed in cardiac tissue. However, in T3-induced hypertrophy, growth is suggested to occur via THRβ1 signalling [13, 22].

To further study the mechanism of the THRβ pathway, we used transgenic mice bearing the Δ337T-*Thrb* mutation. These animals have a point mutation in the ligand-binding domain, rendering THRβ incapable of binding to T3 because it is constitutively associated with corepressors and exerts a potent dominant negative effect on the function of normal THRs [23, 24].

Additionally, microRNAs (miRNAs) are currently being studied as novel cardiac hypertrophy key regulators. miRNAs are approximately 22 nucleotides in length, single-stranded, non-coding RNAs that regulate protein expression through Watson-Crick base pairing between the miRNA 'seed region' and sequences commonly located in the 3' untranslated regions (UTRs) of specific mRNA targets [25]. Noteworthy studies have addressed their potential targets and mechanisms of action in the heart [26-30]. However, little is known about their interaction with THRs in T3-induced cardiac hypertrophy.

Therefore, our study aimed to investigate whether the role of the THRβ isoform in the regulation of T3-induced cardiac hypertrophy involves changes in the expression of specific miRNAs.

Materials and Methods

Ethical Approval

This study was approved by the ethics committee of the Health Sciences Centre, Federal University of Rio de Janeiro (#IBCCF1002).

Animals

Eight-to-twelve-week-old male mice were used. These mice were wild type ($TR\beta^{WT}$) or homozygous ($TR\beta^{\Delta337T}$) for the Δ337T-*Thrb* mutation, and they were generated as previously described, based on a natural human mutation [24]. The genotyping of tail DNA was performed by a mismatched polymerase chain reaction (PCR) as described in our previous study [31]. Mice were maintained in plastic cages and on absorbent bedding under controlled temperature (24±1°C) and lighting (12 h light/dark cycle) conditions, with lights on at 07:00 h. Mice had free access to filtered water and standard chow (Bio-Tec, RJ, Brazil) during all experimental procedures, except during the induction of hypothyroidism, as described below.

A total of 62 $TR\beta^{WT}$ and 41 $TR\beta^{\Delta337T}$ mice were used in all experiments. Thirteen $TR\beta^{WT}$ and 11 $TR\beta^{\Delta337T}$ mice were sacrificed by decapitation, and their sera and hearts were collected and stored at -70°C for baseline evaluation.

Evaluation of mRNA expression in hypo- and hyperthyroidism

From previous works, $TR\beta^{WT}$ and $TR\beta^{\Delta337T}$ mice were shown to present different TH levels at baseline [13, 24, 31]. Therefore, to study gene expression under the same thyroid status, the TH serum concentrations of both genotypes were matched by subjecting the mice to hypothyroidism induction or hyperthyroidism induction. Hypothyroidism was induced by 5 weeks of treatment with 0.15% PTU (5-*propyl-2-thiouracil*; Sigma-Aldrich, SP, Brazil) added to the diet (Hypo). Fifteen $TR\beta^{WT}$ and thirteen $TR\beta^{\Delta337T}$ mice were subject to this PTU treatment. Hyperthyroidism was induced by 3 weeks of daily T3 (*3,5,3´-Triiodo-L-Thyronine*; Sigma-Aldrich, SP, Brazil) subcutaneous (s.c.) injections, at increasing doses of 0.2, 0.5 and 1.0 μg/100 g body weight (bw), after the initial 5 weeks of hypothyroidism induction (Hyper). Hypothyroidism and hyperthyroidism protocols were followed in accordance to recent guidelines published by a group of thyroid experts [32, 33]. Twenty-four hours after the final injection of vehicle or T3, mice were sacrificed by decapitation, and their sera and hearts were collected and stored at -70°C until evaluation.

RNA extraction

Total RNA was extracted from samples (atria and ventricles) by standard methods (TRIzol Reagent; Life Technologies, CA, USA). The total RNA was divided into aliquots for mRNA and miRNA quantification and for miRNA microarray analyses.

Quantification of mRNA

Total RNA was reverse transcribed using 1 μg of RNA and a Reverse Transcription kit (Promega, WI, USA). Quantitative reverse transcription (RT)-PCR was used to analyse the mRNA expressions of α and β myosin heavy chain (*Myh6* and *Myh7*), β1 and β2 adrenergic receptors (*Adrb1* and *Adrb2*), sarcoplasmic reticulum Ca^{++}-ATPase 2a (Serca2, *Atp2a2*) and hyperpolarization-activated cyclic nucleotide-gated channels 2 (*Hcn2*). Real-time RT-PCR was performed using the Master Cycler Realplex system (Eppendorf, Germany) and Maxima SYBR Green/ROX qPCR Master Mix 2X (Fermentas, MA, USA). Intron spanning primers were synthesized by Integrated DNA Technologies (IA, USA) and were obtained from references as shown in Table 1. 36B4 (*Rplp0)* was used as the endogenous control as previously described by us and validated in the heart by others [33-35]. Samples were analysed in duplicate, and the cycle parameters were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 45 s. The product purity was confirmed by agarose gel and melting curve analyses, and the efficiency of each assay was confirmed and accepted when near 100%. Changes in the mRNA expression were calculated from the cycle threshold after correcting for *Rplp0* according to the $2^{-\Delta\Delta Ct}$ method [36]. Data are expressed as fold induction over the control group as stated in the figure legends.

MicroRNA analysis

For the miRNA expression analysis, 10 ng of total RNA was reverse transcribed using a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, CA, USA) and RT primers provided with the miR-1 and miR-208a TaqMan miRNA Assay (Applied Biosystems, CA, USA) according to the manufacturer's

instructions. The miRNA expression was detected from the cDNA product using a TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems, CA, USA) and the TaqMan miRNA Assay. Small nuclear RNA U6 (SNRNA1973, Applied Biosystems, CA, USA) was used for the normalization of data. Amplification and detection were performed using the Master Cycler Realplex system (Eppendorf, Germany). Relative quantifications were calculated according to the Pfaffl method [37].

Microarray analyses were performed by LC Sciences (TX, USA) using 2 µg of total RNA from the wild-type ($TR\beta^{WT}$) or homozygous ($TR\beta^{\Delta337T}$) group at the end of the hyperthyroidism protocol.

Table 1. Primers used in qPCR

Primers	Sequence	
Rplp0 F	TGT TTG ACA ACG GCA GCA TTT	[34]
Rplp0 R	CCG AGG CAA CAG TTG GGT	
Adrb1 F	GCT GCA GAC GCT CAC CA	[59]
Adrb1 R	GCG AGG TAG CGG TCC AG	
Adrb2 F	CAC AGT CAT TGC CAA GTT CG	[59]
Adrb2 R	CGG GCC TTA TTC TTG GTC AGC	
Atp2a2 F	ATA CTG ATG GCA CTT CAC TGG	[60]
Atp2a2 R	AAG ACA GGC ACA CTT ACC AC	
Hcn2 F	GAC AAT TTC AAC GAG GTG CTG	[61]
Hcn2 R	TGA TGG CAT TCT CCT GGT TG	
Myh7 R	ACA TAC TCG TTC CCC ACC TTC	[34]
Myh7 F	AGC TGA CAG GGG CCA TCA T	
Myh6 R	CTC CGG ATT CTC CGG TGA T	[34]
Myh6 F	CCT CCT CAC ATC TTC TCC ATC TCT	

Functional studies

$TR\beta^{WT}$ and $TR\beta^{\Delta337T}$ mice were studied at baseline and under hypo- and hyperthyroid conditions. After 5 weeks of treatment with 0.15% PTU added to food (HYPO), mice received daily s.c. injections for 2 weeks of T3 at 50 µg/100 g bw (HYPER) while maintaining the PTU treatment. At the end of the experimental protocol, mice underwent echocardiography [38] and electrocardiography (ECG) as described below.

Echocardiography analysis

Animals were anesthetized with 1.5% isoflurane gas, dichotomized in the precordium region and examined by Vevo 770 (VisualSonics, OT, Canada) using a 30-MHz transducer. Cardiac geometries and the systolic and diastolic functions of the left ventricles were assessed using one- and two-dimensional modes. The determination of the left ventricular systolic function was established through an analysis of the ejection fraction, which was calculated using a modified Simpson's method [39]. Using the aid of a cursor, the analysis was delimited to the entire left ventricular cavity in systole and diastole. Next, the equipment automatically calculated the stroke and end diastolic volumes, thereby providing the ejection fraction. Doppler echocardiography analysis was performed by a single experienced expert who was unaware of the origin of the animals or the experimental groups to which they belonged. All values were obtained according the recommendations of the American Society of Echocardiography [38].

Electrocardiogram recording

Records were obtained in conscious mice 48 hours after the implantation of electrodes. Records were started 15 min after linking the connector to the data acquisition system (3A9 amplifier; Tektronix/TL-2 A/D Interface; Axon Instruments, CA, USA). The surgical procedures and ECG recordings were carried out as previously described in detail by our group [40].

The hypothyroid and hyperthyroid mice were sacrificed, and the hearts were collected and weighed. The tibia lengths were measured to obtain the relative heart weight [heart weight (mg)/by tibia length [17, 18, 26]], an indirect parameter used to assess cardiac hypertrophy.

Serum hormone measurements

Specific radioimmunoassay [39] commercial kits were used to measure the total triiodothyronine (T3) and the total thyroxine (T4) (MP Biomedicals™, CA, USA) by the solid phase method. Thyroid-stimulating hormone (TSH) expression was measured by the double-antibody method, using NIDDK mouse TSH antiserum (AFP98991), as previously described [41]. All samples were measured in duplicate and within the same assay.

Statistical analysis

Data were reported as the means ± standard errors of the means (S.E.M.). The number of animals is indicated in the figure legends. Student's t-test was employed when comparisons were made for different genotypes, and a two-way analysis of variance (ANOVA) was employed when comparisons were made for

Table 2. Baseline parameters of wild type ($TR\beta^{\Delta337T}$) and homozygous ($TR\beta^{\Delta337T}$) mice. EDD, end-diastolic diameter; RWT, relative wall thickness, end-systolic diameter (2* PWT(d)/EDD); PWT(d), posterior wall thickness N=5-8 animals/group

	$TR\beta^{wt}$	$TR\beta^{\Delta337T}$	
BW (g)	74.3 ± 2.64	78.5 ± 0.95	NS
T4 (μg/dl)	4.09 ± 0.36	>18	P<0.001
PWTs (mm)	0.89 ± 0.01	0.99 ± 0.04	NS
EDD (mm)	3.77 ± 0.25	3.98 ± 0.39	NS
RWT	0.48 ± 0.04	0.51 ± 0.04	NS
Left ventricular mass (mg)	97.8 ± 6.85	127.5 ± 20.8	NS
End-systolic volume (μL)	55.5 ± 2.33	29.8 ± 6.46	P<0.02
End-diastolic (μL)	55.8 ± 2.40	50.5 ± 10.9	NS
Ejection Fraction (%)	68.0 ± 3.24	65.6 ± 3.33	NS
Stroke volume (μL)	37.8 ± 0.85	43.8 ± 6.85	NS

different genotypes and for different treatments. Two-way ANOVAs were followed by Bonferroni multiple comparisons tests for the assessment of significance (Graph Pad Prism 6; GraphPad Software, Inc., CA, USA). Differences were considered to be significant at a P≤0.05.

Results

Cardiac baseline gene expression

First, mice were studied at baseline. No differences were observed in weight, but the total serum T4 concentration was four times higher in the $TR\beta^{\Delta337T}$ mice than in the $TR\beta^{WT}$ mice (Table 2). There were no differences in the ventricular mRNA expressions of *Myh6* and *Myh7* between the $TR\beta^{WT}$ and $TR\beta^{\Delta337T}$ mice (Fig. 1B). However, the ventricular mRNA expressions of *Adrb1* and *Adrb2* were significantly lower and higher, respectively (P<0.01 and P<0.05) in the $TR\beta^{\Delta337T}$ mice compared with the $TR\beta^{WT}$ mice (Fig. 1A). The $TR\beta^{\Delta337T}$ mice also showed significantly lower *Atp2a2* ventricular mRNA expression (P<0.001) and significantly greater *Hcn2* atrial mRNA expression (P<0.01) compared with the $TR\beta^{WT}$ mice (Fig. 1A). As previously shown, at baseline, the $TR\beta^{\Delta337T}$ mice presented increased levels of T4 (Table 1) and T3 [13, 14, 24, 42-44]. Therefore, the observed differences seen here do not reflect the actual TH levels.

Cardiac gene expression after hypo- and hyperthyroidism induction

Mice were subjected to hypo- and hyperthyroidism induction to evaluate the role of THRβ in the absence and presence of T3 upon gene expression.

After PTU treatment, both groups presented undetectable serum concentrations of T4 (data not shown), decreased levels of total T3 ($TR\beta^{WT}$ Hypo: 45.4 ± 8.6 and $TR\beta^{\Delta337T}$ Hypo:

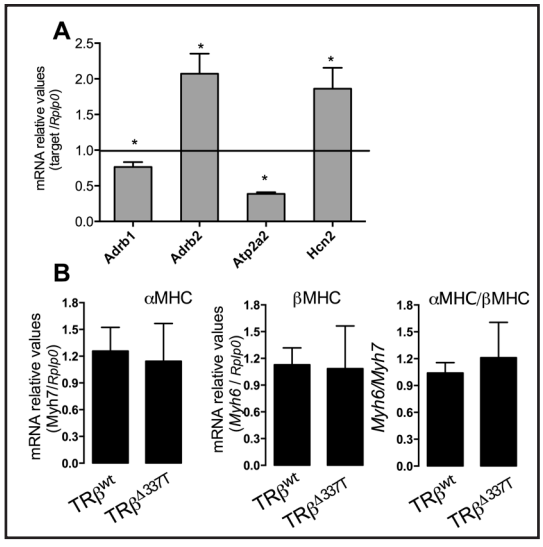

Fig. 1. Baseline parameters of wild-type ($TR\beta^{WT}$) and homozygous ($TR\beta^{\Delta337T}$) mice. (A) Ventricle mRNA expression of AR-β1 (*Adrb1*), AR-β2 (*Adrb2*), and SERCA2 (*Atp2a2*) and atrial mRNA expression of HCN2 (*Hcn2*) in nine $TR\beta^{wt}$ and five $TR\beta^{\Delta337T}$mice. (B) Ventricle mRNA expression of α-MHC (*Myh6*) and β-MHC (*Myh7*) and the α-MHC/β-MHC (*Myh6/Myh7*) ratio. The data are shown as the means of fold change ± S.E.M. and represent the averages of three independent experiments. Differences were considered significant when P<0.05. Line represents the expression level of $TR\beta^{WT}$.

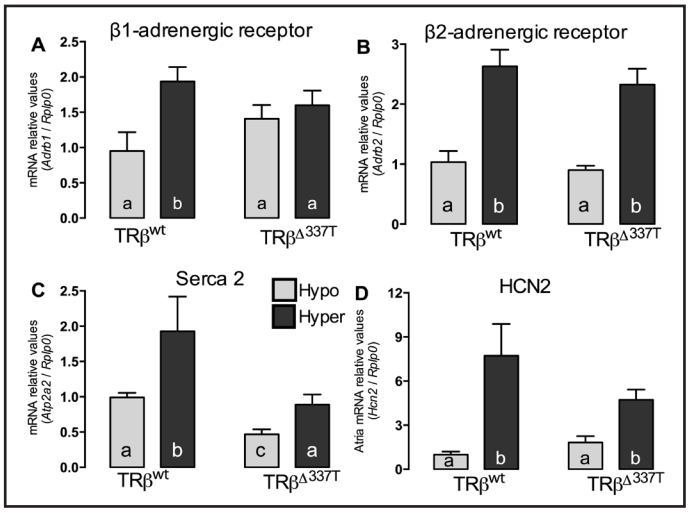

Fig. 2. Effect of thyroid hormone (TH) deficiency and excess on classic TH-responsive genes in the heart. (A-C) Ventricular mRNA expression of AR-β1 (*Adrb1*), AR-β2 (*Adrb2*) and SERCA2 (*Atp2a2*) and (D) atrial mRNA expression of HCN2 (*Hcn2*) in TRβ^{wt} and TRβ$^{\Delta 337T}$ mice, according to qPCR analysis. The data are shown as the means of fold change ± S.E.M. Data were normalized for each mRNA level relative to the hypothyroid TRβ^{wt} group. Same letters indicate similar means. Six to eight animals were evaluated in each group. Hypo PTU-treated mice, Hyper: PTU+T3-treated mice.

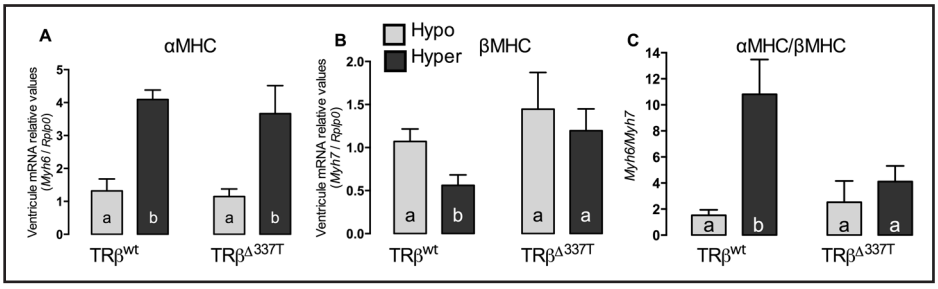

Fig. 3. Ventricular mRNA expression of α-MHC (*Myh6*, A) and β-MHC (*Myh7*, B) in TRβ^{WT} and TRβ$^{\Delta 337T}$ mice according to qPCR analysis. (C) α-MHC/β-MHC (*Myh6*/*Myh7*) ratio. The data are shown as the means of fold change ± S.E.M. Data were normalized for each mRNA level relative to the hypothyroid TRβ^{wt}. Same letters indicate similar means. Six to eight animals were evaluated in each group. Hypo: PTU-treated mice, Hyper: PTU+T3-treated mice.

40.0 ±8.7 ng/dL) and, importantly, elevated levels of TSH (above threshold detection; data not shown). After T3 treatment, TRβ^{WT} and TRβ$^{\Delta 337T}$ presented similar levels of T3 (78.8 ± 4.5 and 100.6 ± 9.0 ng/dL respectively). *Thra* and *Thrb* mRNA were also evaluated. No differences were seen regarding *Thrb* mRNA however, T3 treatment caused a decreased in Thra mRNA in both groups (data nor shown).

As expected, the TRβ^{WT} mice presented significantly greater mRNA ventricular expression levels of *Adrb1* and *Atp2a2* after T3 treatment (P<0.05); however, these effects were not significantly different in the TRβ$^{\Delta 337T}$ mice (Fig. 2A and C). Both mice groups presented significantly higher *Adrb2* and *Hcn2* mRNA expression levels (P<0.001) after T3 treatment (Fig. 2B and D). In addition, both the TRβ^{WT} and TRβ$^{\Delta 337T}$ mice groups presented significantly higher *Myh6* mRNA expression levels after T3 treatment (Fig. 3A, P<0.01). However, only the TRβ^{WT} mice showed a significant reduction in *Myh7* (P<0.05) after T3 treatment (Fig. 3A and B). Thus, as shown in Fig. 3C, there was an increase in the α/β-MHC ratio in the TRβ^{WT} mice when comparing between the Hyper and Hypo groups (P<0.01). A similar increase was not observed in the TRβ$^{\Delta 337T}$ mice.

Cardiac morphology and function assessment

After the T3 treatment, the EDD did not change in the TRβ^{WT} mice compared with the hypothyroid mice, a phenomenon that was not observed in the TRβ$^{\Delta 337T}$ mice (Table 3); on the contrary, EDD increased after T3 treatment. Although the PWT (d) and RWT were not significantly different among the groups, the RWT thickness increased in the TRβ^{WT} mice and decreased in the TRβ$^{\Delta 337T}$ mice (P=0.06). These observed opposite effects induced by the T3 treatment resembled the concentric and eccentric hypertrophy in the TRβ^{WT} and TRβ$^{\Delta 337T}$

Table 3. Parameters recorded from the echocardiogram of wild-type ($TR\beta^{WT}$) and homozygous ($TR\beta^{\Delta337T}$) hypothyroid and hyperthyroid mice. EDD, end-diastolic diameter; RWT, relative wall thickness, end-systolic diameter (2* PWT(d)/EDD); PWT(d), posterior wall thickness.*P<0.05 compared to hypothyroid $TR\beta^{\Delta337T}$. n=3-11 animals/group

	$TR\beta^{wt}$		$TR\beta^{\Delta337T}$	
	HYPO	*HYPER*	*HYPO*	*HYPER*
EDD (mm)	3.47 ± 0.13	3.95 ± 0.40	3.84 ± 0.16	4.24 ± 0.13*
RWT	0.63 ± 0.07	0.71 ± 0.27	0.54 ± 0.05	0.35 ± 0.02
PWT(d) (mm)	1.10 ± 0.09	1.24 ± 0.28	1.09 ± 0.12	0.87 ± 0.11

Table 4. Parameters recorded from the electrocardiogram (ECG) of wild-type ($TR\beta^{\Delta337T}$) and homozygous ($TR\beta^{\Delta337T}$) HYPO and HYPER group mice. *P<0.05. N=5 animals/group

	$TR\beta^{wt}$		$TR\beta^{\Delta337T}$	
	HYPO	*HYPER*	*HYPO*	*HYPER*
P-wave (ms)	12.93 ± 0.49	8.02 ± 0.13*	12.03 ± 1.08	11.64 ± 0.68
PR Interval (ms)	32.78 ± 1.65	27.84 ± 1.96	33.60 ± 1.45	29.42 ± 1.54
QRS Duration (ms)	19.75 ± 1.92	19.53 ± 2.12	21.66 ± 1.04	17.78 ± 1.13
QT Interval (ms)	30.13 ± 2.40	27.13 ± 0.24	30.02 ± 2.92	24.28 ± 3.64
T-Wave (ms)	18.50 ± 1.82	14.63 ± 0.84	19.18 ± 2.91	12.92 ± 3.20
Heart Rate (bpm)	528.7 ± 29.49	655.8 ± 22.86*	553.4 ± 51.79	677.6 ± 45.94*

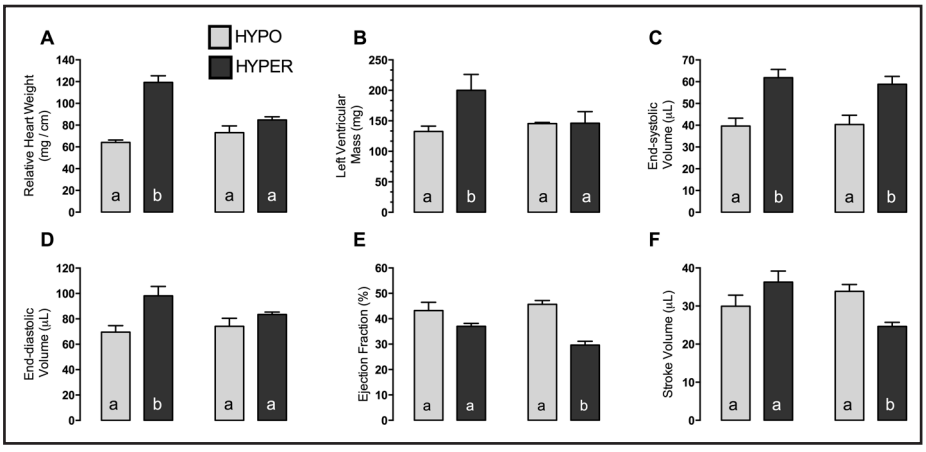

Fig. 4. Cardiac function assessment, by echocardiography [38], of $TR\beta^{WT}$ and $TR\beta^{\Delta337T}$ mice. (A) Relative heart weight (mg/cm); (B) left ventricular mass (mg); (C) end-systolic volume (µL); (D) end-diastolic volume (µL); (E) ejection fraction (%); (F) stroke volume (µL). HYPO: PTU-treated mice, HYPER: PTU+T3-treated (higher dose) mice. See details in the Material and Methods section. The data are shown as the means ± S.E.M. of at three independent experiments. Three to eleven animals were evaluated in each group. Same letters indicate similar means.

mice, respectively. As expected, the relative heart weight (P<0.001) and the left ventricular mass (P<0.05) significantly increased only in the $TR\beta^{WT}$ mice after the T3 treatment (Fig. 4A and B). The $TR\beta^{\Delta337T}$ mice did not present with these changes, confirming the dilated hypertrophy. Any potential changes to cardiac contractility induced by these two different cardiac remodelling processes were assessed by examining the systolic function in the echocardiograms. A significant decrease in the systolic function was observed only in the $TR\beta^{\Delta337T}$ mice treated with T3, accompanied with a significant reduction in the ejection fraction and stroke volume (Fig. 4E, P<0.001; and 4F, P<0.05). These results suggest that the $TR\beta^{\Delta337T}$ mice developed heart failure in response to high T3 and the $TR\beta^{WT}$ mice, as expected, did not.

ECG analysis

After the T3 treatment, the P-wave duration was significantly reduced in the $TR\beta^{WT}$ mice (P<0.05). However, this decrease was not observed in the $TR\beta^{\Delta337T}$ mice (Table 4). The heart rate was significantly increased in both T3-treated groups (P<0.05).

Analysis of microRNA

To investigate possible mechanisms involved in T3-induced cardiac hypertrophy, we first studied the ventricular expression of two well-known microRNAs, miR-1 and miR-208a, under the hypo- and hyperthyroid states (Fig. 5A and B). The expression of miR-1 significantly increased in the TRβ^{WT} and TRβ$^{\Delta 337T}$ mice after T3 treatment (P<0.05; Fig. 5A). However, miR-208a ventricular expression significantly increased only in the T3-treated TRβ^{WT} mice (P<0.05, Fig. 5B).

We further investigated the broader miRNA profiles of these two groups. Unexpectedly, we found only a small number of miRNAs that were differently expressed in these groups (Fig. 6). Only nine miRNAs were differentially expressed (p<0.05). Four miRNAs were down-regulated (miR-199a-3p, miR-346-3p, miR-92b-3p, and miR-6516-5p) and five were up-regulated (miR-568, miR-703, miR-713, miR-1231-5p, and miR-7219-3p) in the TRβ$^{\Delta 337T}$ mice compared with the TRβ^{WT} mice. We next used Ingenuity Systems Pathways Analysis software (IPA; Ingenuity Systems, CA, USA) to analyse *in silico* the significant differentially regulated miRNAs. Among the nine miRNAs analysed, only miR-199a-3p is known to be related to cardiac hypertrophy.

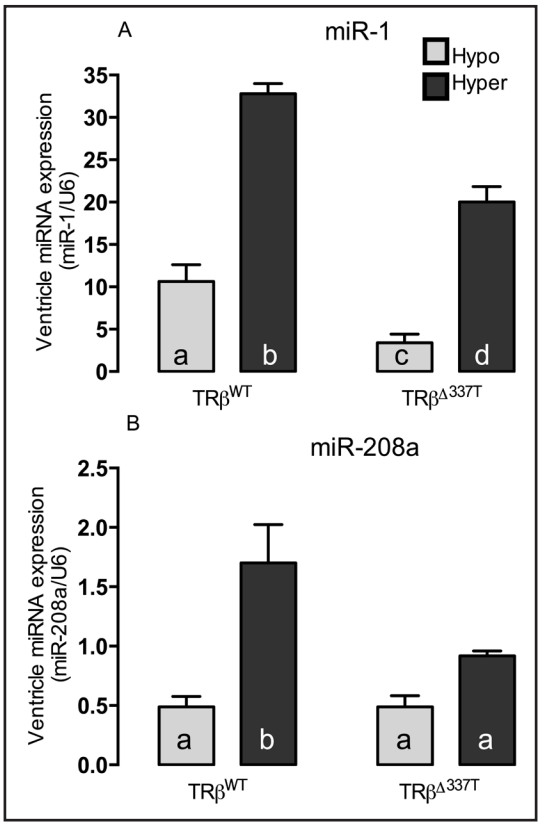

Fig. 5. Ventricular microRNA expression in TRβ^{WT} and TRβ$^{\Delta 337T}$ mice according to qPCR analysis. (A) miR-1; (B) miR-208a expression. The data are shown as the means of fold change ± S.E.M. Data were normalized for each miRNA level relative to the hypothyroid TRβ^{wt}. Same letters indicate similar means. Three animals were evaluated per group. HYPO: PTU-treated mice, HYPER: PTU+T3-treated mice.

Discussion

This study evaluated *in vivo* and *in silico* the hypertrophic effects of thyroid hormone receptor β (*Thrb*) on the expressions of key genes that are involved in the control of cardiac function and known to be directly regulated by T3 [19, 20, 22, 45-48]. We showed that THRβ plays a fundamental role in cardiac hypertrophy induced by TH (Fig. 7). Concentric cardiac hypertrophy expected after T3 treatment was confirmed by the significant increases in the relative heart weight and the left ventricular mass and relative wall thickness in the TRβ^{WT} mice, as indicated in pathology and echocardiograms. The absence of a functional THRβ leads to the dilated cardiomyopathy in the TRβ$^{\Delta 337T}$ mice in response to high T3 levels (Fig. 7). Different from the TRβ^{WT} group, which maintained systolic function at physiological levels, the mutant animals showed decreased

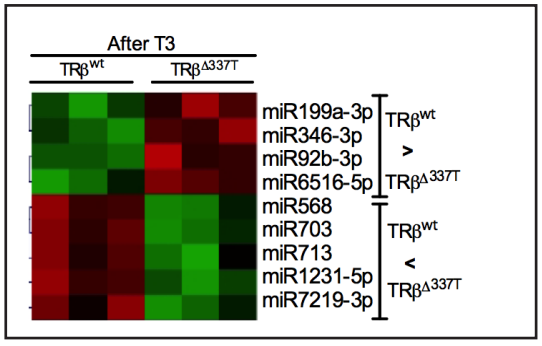

Fig. 6. Differentially expressed miRNAs in the ventricles of T3-treated TRβ^{WT} and TRβ$^{\Delta 337T}$ mice. Mouse miRNA was analysed by LC Sciences (Houston, TX). Nine miRNAs were differentially expressed (P<0.05): four miRNAs were down-regulated, and five were up-regulated in TRβ$^{\Delta 337T}$ mice compared with TRβ^{WT}. Three animals were evaluated per group.

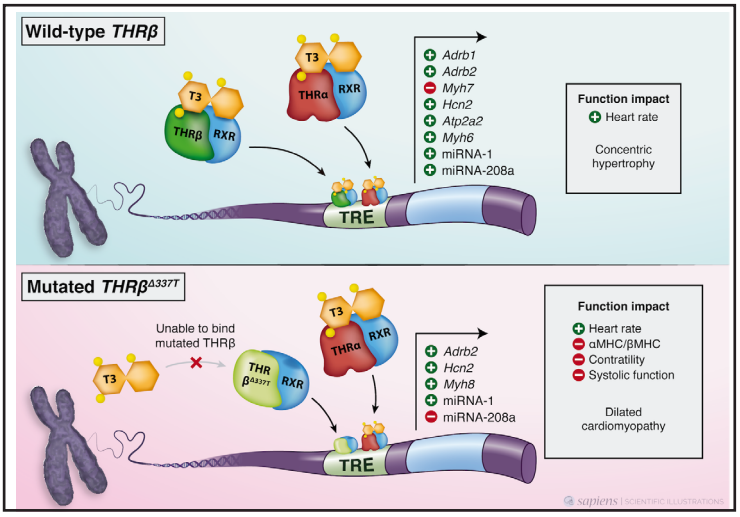

Fig. 7. Schematic representation of proposed role of THRβ in T3-induced cardiac concentric hypertrophy. *Myh6*: α myosin heavy chain, *Myh7*: β myosin heavy chain, *Adrb1*: β1 adrenergic receptor, *Adrb2*: β2 adrenergic receptor, *Atp2a2*: sarcoplasmic reticulum Ca^{++}-ATPase 2a, *Hcn2*: hyperpolarization-activated cyclic nucleotide-gated channels 2, TRE: thyroid hormone response element.

contractility and signs of heart failure as assessed by the echocardiographic exam. We and others have previously suggested that the *Thrb* mutation in heterozygotes could prevent an increase in the left ventricular mass induced by T3 [13, 45], indicating that blocking the binding of T3 to THRβ1 somehow interfered with the induction of cardiac concentric hypertrophy. To definitively demonstrate this mechanism, we analysed $TR\beta^{\Delta 337T}$ mice.

Because the $TR\beta^{\Delta 337T}$ mice presented elevated serum concentrations of T4 and T3 [13, 14, 24, 42-44], it was necessary to normalize these hormone levels to investigate whether the changes found in the baseline gene expression were due to the absence of T3 binding to THRβ or to the increased THRA signalling. Additionally, as described below, a complex pattern of responses was observed where both isoforms of THRs had specific roles.

The hypertrophy induced by T3 in the WT mice was associated with a possible adaptive response, resulting in the stabilization of systolic function, whereas the $TR\beta^{\Delta 337T}$ mice exhibited clear contractile deficits as assessed by echocardiography (Fig. 4). The increased mass of the left ventricle exhibited by the T3-treated $TR\beta^{WT}$ mice was likely essential to maintain a normal cardiac output required by the high metabolic demand associated with hyperthyroidism. Moreover, the lack of functional T3/THRβ in the $TR\beta^{\Delta 337T}$ HYPER group resulted in dilated cardiomyopathy and resulted in a clear reduction of systolic function (assessed in terms of ejection fraction and stroke volume) (Fig. 4). It is important to point out that T3 can bind normally to THRα1 in both groups.

A systemic effect caused by T3 bound to THRα1 (e.g., decreased afterload) in the maintenance of cardiac performance observed in the $TR\beta^{WT}$ HYPER group should not be ruled out. It is possible that $TR\beta^{\Delta 337T}$ HYPER mice were deprived of this effect, which would further increase the importance of THRβ in the regulation of systemic vascular resistance in the $TR\beta^{\Delta 337T}$ HYPER group.

The contribution of the THRA isoform to the T3-induced cardiac hypertrophy was suggested in a previous study, wherein the over-expression of *Thra1* in cardiomyocytes resulted in a hypertrophic phenotype, even in the absence of T3. THRβ1 played an inhibitory role in the activation of p38 MAPK and, hence, cardiac hypertrophy [49]. In addition, after a T3 treatment in a primary culture of rat cardiomyocytes, Kenessey and Ojamma described the involvement of THRα1 in a direct protein-protein interaction with PI3K in the cytosol, activating mTOR and promoting protein synthesis and cardiac hypertrophy [50]. The differences in the models and protocols may account for a few of the differences between our data and those found in the literature regarding the importance of different THR isoforms for T3-induced cardiac hypertrophy. Kinugawa's group used a *Thra* overexpression model, which may amplify (supraphysiologically) the T3/THRα signalling and overestimate the contribution of this pathway. In addition, although the involvement of THRα was unequivocally demonstrated for hypertrophy in both studies [49, 50], the studies used

cardiomyocyte cultures, which limit the physiological extrapolation of their results to the whole animal.

Kinugawa and colleagues have suggested that the expression of THRs in cardiac tissue was linked to the type of hypertrophic stimulus [49]. According to these authors, the THRβ isoform would be responsible for mediating a remodelling response because it was expressed at higher levels in cultures of cardiomyocytes treated with T3 and *in vivo* exercising animals. However, the THRα isoform would be responsible for mediating the pathological response because its expression was increased in the pathological cardiac hypertrophy models [51]. Thus, the mediation of cardiac hypertrophy by different receptor isoforms was not necessarily mutually exclusive with our findings and those of others [13, 45].

The increased α-MHC/β-MHC ratio induced by the T3 treatment in the $TR\beta^{WT}$ mice was not observed in the $TR\beta^{\Delta337T}$mice. A reduced α-MHC/β-MHC ratio may result in reduced ATPase activity and, thus, a lower contractile velocity in these animals. The down-regulation of *Myh7* by T3 was impaired in the $TR\beta^{\Delta337T}$ mice (Fig. 3B), whereas *Myh6* responded normally to T3 (Fig. 3A). The decreased α-MHC/β-MHC ratio (Fig. 3C) impaired the contractility profile, contributing to a drop in the ejection fraction and the stroke volume of the $TR\beta^{\Delta337T}$ animals, as observed in the echocardiograms (Fig. 4E and F).

Clearly, the T3 stimulation of *Adrb1* mRNA was dependent on THRβ because there was a significant reduction in its expressions in the $TR\beta^{\Delta337T}$ mice between baseline levels (Fig. 1A) and after the T3 treatment (Fig. 2A). Similarly, THRβ is important in the regulation of *Atp2a2* mRNA expression, as previously described (Fig. 1A and 2C) [51]. Moreover, when comparing the $TR\beta^{WT}$ and $TR\beta^{\Delta337T}$ groups, regarding the absence or presence of T3, we observed that the $TR\beta^{WT}$ animals had higher *Atp2a2* mRNA expression levels than the $TR\beta^{\Delta337T}$ animals, which may be reflected later in lower cardiac functional diastolic capacities, as was indeed observed in the echocardiograms (Fig. 2D and 4D). However, we cannot neglect the contribution of the *Adrb1* receptors to the modulation of SERCA2, considering the lower expression of this receptor in the $TR\beta^{\Delta337T}$ mice. It is plausible to assume that the changes observed in *Atp2a2* mRNA expression are due, at least in part, to decreased β1-adrenergic signalling, either at baseline (Fig. 1A) or after T3 stimulation (Fig. 2C), which is also mediated by THRβ.

Adrb2 and *Hcn2* mRNA expressions are regulated by T3 independently of THRβ (Fig. 2B and D) suggesting an important role of THRα1. HCNs are the channels responsible for the I_f (funny) current that permits cation (Na^+ and K^+) passage when stimulated by plasma membrane hyperpolarization. The I_f current is the main component of the slow diastolic depolarization phase of the action potential of sinus node cells. Even small reductions in HCN2 are sufficient to cause severe bradycardia [20]. The increase in *Hcn2* mRNA atrial expression at baseline was expected (Fig. 2D) because we have previously observed that $TR\beta^{\Delta337T}$ mice exhibit significant increases in heart rate [52] under baseline conditions compared with $TR\beta^{WT}$ animals with a cardiac phenotype very similar to hyperthyroidism. This increased HR occurs because the $TR\beta^{\Delta337T}$ animals have high serum concentrations of TH, which interact with THRα1. Thus, the presence of THRα1 results in an increased expression of *Hcn2*, which contributes to the increased HR and accounts for sinus tachycardia, a very common clinical sign in patients with RTH [53]. The increased HR in the $TR\beta^{WT}$ and $TR\beta^{\Delta337T}$ mice after T3 treatment, as observed by ECG (Table 3), is associated with the *Hcn2* mRNA atrial expression observed here (Fig. 2D) and agrees with previous studies that demonstrated that HCN2 and heart rate regulation occur via THRα and not via THRβ [20].

ECG also demonstrated a significant reduction of the P-wave duration in normal mice treated with T3 compared with hypothyroid mice. This effect of the T3 reducing the P-wave was absent in the $TR\beta^{\Delta337T}$ mice (Table 3), suggesting an important role for THRβ in the atrial conduction of these animals. Our group previously described that the up-regulation of Connexin 40 atrial gene expression exerted by TH was blocked by the over-expression of a non-functional dominant-negative THRβ in the heart [40]. However, when THRβ functionality was systemically compromised, as in our current transgenic animal model, the expression of Connexin 40 did not seem to be involved because there was no significant alteration in its expression in the $TR\beta^{\Delta337T}$ animals (data not shown). Thus, further studies

are required to better understand the altered atrial conduction exhibited by TRβ$^{\Delta 337T}$ mice after T3 treatment.

Finally, to investigate the possible mechanisms involved in THRβ-mediated cardiac hypertrophy, we evaluated the expression of two miRNAs: miR1, a muscle-specific miRNA expressed both in striated skeletal and cardiac muscles [54], and miR208a, which is exclusively expressed in cardiomyocytes [55]. Our analysis showed that, in cardiac tissue, TH up-regulates miR-1 expression, and this action occurs independently of THRβ because the same up-regulation was observed in both mice (Fig. 5A). It has been demonstrated that miR-1 attenuates cardiac hypertrophy [56]. However, in our model, there was no correlation between hypertrophy and miR-1 expression. Regarding miR-208a, we confirmed its up-regulation by T3 [28, 29, 57] and demonstrated that this effect was mediated by THRβ because T3 could not increase miR-208a in TRβ$^{\Delta 337T}$ mice (Fig. 5B). It has been reported that miR208a facilitates hypertrophic growth because it represses the expression of cardiac hypertrophy inhibitory proteins, such as myostatin and thyroid hormone-associated protein 1 (THRAP1) [29]. Thus, the findings that miR-208a induces hypertrophy and that its expression was similar to the profile observed for the relative heart weight (Fig. 4A) and ventricular mass (Fig. 4B) confirmed that miR-208a is involved in the induction of cardiac hypertrophy. Thus, when the binding of THRβ to T3 was blocked, the expected up-regulation of miR-208a was prevented. In turn, miR-208a was unable to repress the transcription factors inhibiting cardiac hypertrophy, resulting in the impairment of cardiac hypertrophy. Because very few studies exist concerning the involvement of THRs and miRNAs in T3-induced hypertrophy [30, 58], we evaluated the differences in the miRNA profiles of both groups after T3 treatment. Recently, Janssen *et al.* [58] found more than 50 differentially expressed mRNAs when hypothyroid mice were treated with an extremely high dose of T3 for 3 days. Surprisingly, we found only nine differently expressed miRNAs, indicating the specific role of THRβ in this regulation. After an IPA analysis, only miR-199a-3p, which was down-regulated in the TRβ$^{\Delta 337T}$ mice, was implicated in hypertrophy mechanisms. Although very little is known about the function of miR-199a-3p in the heart, it was shown that it is present in mice, rats, dogs and monkeys and in different cell types [27]. Although further investigation concerning the specific role of miR-208a and miR-199a-3p exceeds the scope of our work, this knowledge will be useful in the future because miRNAs can be used in therapeutic approaches to avoid damage in the heart of patients with increased levels of TH. In conclusion, as shown in Fig. 7, we suggested a crucial role for the regulation of specific mRNA and miRNA in T3-induced adaptive cardiac hypertrophic growth and alteration in heart functions mediated by THRβ.

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Disclosure Statement

No conflicts of interest, financial or otherwise, are declared by the authors.

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