Effects of hyper- and hypothyroid on expression of thyroid hormone receptor mRNA in rat myocardium

C R Liu¹, L Y Li, F Shi, X Y Zang, Y M Liu¹, Y Sun and B H Kan

Key Laboratory of Hormones and Development, Ministry of Health China, Endocrinology Institute of Tianjin Medical University, Tianjin Medical Institute, Tianjin 300070, China

¹Department of Pathology, Medical College of Chinese People's Armed Police Force, Tianjin 300162, China

(Correspondence should be addressed to L Y Li; Email: lily@tjmu.edu.cn)

Abstract

Thyroid dysfunction is classified into hyperthyroidism and congenital hypothyroidism (CH). Both hyperthyroidism and CH can cause heart lesions; however, the mechanisms involved remain unclear. The left ventricle was collected from eu-, hyper-, and hypothyroid rat. RNA was extracted and reverse-transcribed to cDNA. Real-time fluorescence quantitation-PCR was used to quantify the differential expression of thyroid hormone receptor (TR) subtype mRNA among eu-, hyper-, and hypothyroid rat myocardium. Here, we show that compared with the normal myocardium, TR α 1 mRNA expression was upregulated by 51% ($P < 0.01$), TR α 2 mRNA expression was downregulated by 58% ($P < 0.01$), and TR β 1 mRNA expression remained unchanged in hyperthyroid rat myocardium ($P > 0.05$). TR α 1, TR α 2, and

TR β 1 were expressed in normal and hypothyroid rat myocardium throughout the developmental process. In hypothyroid rats, myocardial TR α 1 mRNA expression was generally downregulated and the expression peak appeared late. Myocardial TR α 2 mRNA expression was generally upregulated and the expression peak appeared late. Myocardial TR β 1 mRNA expression was generally downregulated and changed similarly with the control group. In addition, the hypogenetic myocardium can be seen in the hypothyroid rat by pathology study. Taken together, the abnormal expression of TR subtype mRNA may have a close relationship with the pathogenesis of CH and hyperthyroidism heart disease.

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Introduction

The heart is a major target organ for thyroid hormone (tri-iodothyronine, T₃) action. Thyroid hormone exerts its biological effects largely by influencing thyroid hormone-regulated gene expression via interactions with the high-affinity thyroid hormone receptor (TR) located in the nucleus. TR plays an important role in mediating the cardiac physiologic actions of T₃ (Sussman 2001, Dillmann 2002).

Normal thyroid hormone level is essential for maintaining normal heart structure and function. In clinical settings, patients with hyperthyroidism or congenital hypothyroidism (CH) may present abnormalities in myocardial structure and heart function. Changes in thyroid status exert marked influences on the contractile and electrophysiological function of the heart. The molecular basis of alterations in electrophysiological function caused by changes in different thyroid hormone status has not been completely explored.

Besides aggravating preexisting ischemic heart disease or other types of heart disease, hyperthyroidism itself may cause atrial fibrillation, cardiac dilatation, heart failure, angina pectoris, or myocardial infarction. The incidence of hyperthyroid heart disease was 10 to 20% among hyperthyroidism patients. Hyperthyroidism is frequently complicated by arrhythmia and,

most commonly by atrial fibrillation (Klein & Ojamaa 2001). Current studies have demonstrated the possible association of atrial fibrillation with Na⁺–K⁺–ATPase. The maintenance of atrial fibrillation depends on the formation of multiple reentrant cycles. In hyperthyroidism, the activity of Na⁺–K⁺–ATPase is enhanced in myocardial cells, which promotes Na⁺ outflow and K⁺ inflow and thus shortens the duration of action potential of individual atrial muscle cell (Rensma *et al.* 1988, Wijffels *et al.* 1995, Maixent *et al.* 2000, Workman *et al.* 2003). In addition, shortening of the action potential duration in the ventricular muscle and concomitant tachycardia may render the ventricle susceptible to developing heart failure known as thyrotoxic heart (Klein 1990, Polikar *et al.* 1993). Hyperthyroidism that has been existing for a long time and has not been treated can result in marked morphological changes of the heart, including atrial or ventricular dilatation, heart weight gain, hypertrophy of myocardial cells, widened spaces between the muscle fibers of the heart (Henle's fissures), and ultimately necrosis of myocardial cells and heart failure. The factors below may be associated with these myocardial pathologies: 1) hyperthyroid symptom is similar to sympathetic nerve system. Furthermore, tachycardia, increased pulse pressure, higher cardiac output, and oxygen wastage caused by hyperthyroidism can be successfully alleviated using sympathetic nerve or β -receptor retarder. Therefore, it was

estimated that hyperthyroid myocardium injury may be related with sympathetic-adrenalin system (Morgan & Baker 1991). 2) In hyperthyroidism, the renin-angiotensin-aldosterone system (RAAS) is activated, the plasma rennin activity is enhanced, the concentrations of angiotensinogen and angiotensin (AT) are increased, and the density of AT receptor in the heart is upregulated. Activation of the RAAS adversely influences the cardiovascular system: on the one hand, it increases blood volume and the heart's burden and, on the other, AT binds to high-affinity AT receptors on myocardial cells or vascular smooth muscle cells and stimulates *c-fos* gene transcription in the nucleus through the intracellular signal transduction system. The *c-fos* protein can promote growth of myocardial or vascular smooth muscle cells and lead to myocardial hypertrophy. As a result, compensated myocardial function turns decompensated followed by heart failure (Katz 1990, Morgan & Baker 1991, Marchant *et al.* 1993, Sernia *et al.* 1993, Basset *et al.* 2001). These findings were derived from biochemical research on the pathogenesis of hyperthyroid heart disease. As for molecular research on the pathogenesis of hyperthyroid heart disease, thyroid hormone-regulated genes have been the major concern. It was demonstrated that myocardial genes upregulated by thyroid hormone include HCN2, HCN4, myosin heavy chain α (MHC α), and sarcoplasmic reticulum calcium-activated ATPase2 (SERCA2), and myocardial genes downregulated by thyroid hormone include MHC β and Na⁺/Ca²⁺ exchanger (Dillmann 1990, 1996, Kahaly & Dillmann 2005). Despite the aforementioned findings, TR, a factor closely associated with the physiologic functions of thyroid hormone, has not been fully investigated and few studies of the association between hyperthyroid heart disease and TR subtypes have been reported.

CH is a relatively common newborn endocrine disorder that occurs with a frequency of $\sim 1:3500$. CH may cause cretinism and myxedema in different age groups. During the fetal and infantile periods, inadequate thyroxine derived from the maternal body or synthesized may cause CH (cretinism). The patient usually presents with myocardial structural developmental abnormalities and serious heart dysfunction, a condition called hypothyroid heart disease. Adult hypothyroid heart disease was first reported by Zondek in 1918. In CH patients, heart damage is very common. It is now thought that 70–80% of hypothyroidism patients have cardiovascular lesions, typically hypothyroid coronary heart disease, hydro-pericardium, cardiomyopathy, or pericarditis with cardiomyopathy (Balducci *et al.* 1991, Di Meo *et al.* 1994, Chao *et al.* 1997). Many patients visit the cardiovascular departments because of heart damages as initial manifestations. Due to diversified clinical manifestations and a chronic disease course, it is very easy to misdiagnose CH as another disease. Hence, to deepen the understanding of the pathogenesis of hypothyroid heart damages is important to increase the life quality of hypothyroid patients. To date, there have been only some pathomorphological reports on hypothyroid heart disease. Nevertheless, few studies of the pathogenesis of hypothyroid heart disease have been reported. In particular, the effect of

CH on myocardial TR subtype mRNA expression at different developmental stages has not yet been reported.

Taken together, thyroid hormone exerts crucial regulatory actions on maintaining normal relaxation and contraction and electrophysiology of the heart. Abnormal thyroid hormone concentrations may lead to myocardial structural and functional abnormalities. Thus far, the pathogenesis of hyper- and hypothyroid heart disease has not been completely elucidated. TR plays an important role in mediating the physiologic actions of thyroid hormone. Whether abnormal thyroid hormone concentrations influence myocardial expression of TR, and whether the pathogenesis of hyper- and hypothyroid heart disease is associated with TR are issues worthy of investigation. It is believed that unraveling these issues will open a new avenue to clinical treatment of these disorders. In order to explore the association between TR and heart lesions during thyroid dysfunction and to provide a new therapeutic strategy for hyper- and hypothyroid heart disease at the molecular level, differential expressions of TR subtype mRNAs among normal, hyperthyroid, and hypothyroid rat myocardium were analyzed using fluorescence quantitation-PCR (FQ-PCR).

Materials and Methods

Experimental animals

All animals were obtained from the Experimental Animal Centre of the Academy of Military Medical Sciences of China. They were raised in a temperature-controlled room (21–23 °C) under a 12 h light:12 h darkness cycle. Food and water were freely available.

Hyperthyroid animals Sixteen Wistar rats (specific pathogen-free animals, weighing 150–180 g) were equally randomized to the control group and the experimental group ($n=8$). Thyroxine tablets (Shanghai Great Wall Pharmaceutical, Shanghai, China) were ground and diluted with normal saline into a 40 g/l suspension. Each rat from the experimental group and the control group (Morreale de Escobar *et al.* 1988) was intragastrically administered 1 ml suspension and normal saline respectively, once a day, for 3 successive weeks. After 3 weeks of treatment, the animals were weighed and anesthetized with 1% pentobarbital, followed by thoracotomy and blood sampling from the heart. Blood samples were centrifuged and the supernatants were obtained for detecting serum free T₃ (FT₃) and free thyroxine (FT₄) levels by RIA (completed by radiology Institute of Tianjin Medical University). The heart was obtained and blood in the cardiac chamber was washed out quickly with diethylpyrocarbonate (DEPC) water. The heart was wiped dry with a sheet of filter paper, weighed, and stored in liquid nitrogen.

Hypothyroid animals Twelve pregnant Wistar rats (specific pathogen-free animals) were equally randomized into the experimental group and the control group. From the 15th day of gestation, each rat from the experimental group was

intragastrically administered with 2.5 ml of 1% propylthiouracil (Dr Herbrand KG, Chem-Pharm. Works, D-77723 Gengenbach, Germany) daily until postnatal 15-day. Litters were CH rats (Gilbert & Paczkowski 2003). In contrast, from the 15th day of gestation, each rat from the control group was intragastrically administered with 2.5 ml normal saline daily until postnatal 15 days. At postnatal days 0 (P0d), 14 (P14d), 21 (P21d), and 45 (P45d), the rats were weighed and killed, and their blood samples were collected from the heart. Serum FT₃ and FT₄ levels were dynamically determined by RIA (completed by radiology Institute of Tianjin Medical University). The heart was obtained and blood in the cardiac chambers was washed out quickly with DEPC water. The heart was wiped dry with a sheet of filter paper, weighed, and stored in liquid nitrogen. There were 40 neonate rats in total and each subgroup contained 5 of them ($n=5$).

Total RNA extraction

Total RNA was isolated from the left ventricle (because this part of the heart makes the major contribution to total cardiac RNA; Gloss *et al.* 2001) with Trizol reagent, as described by the manufacturer (Gibco, Invitrogen Corporation). Total RNA was quantified by absorbance at 260 nm. The integrity of RNA was assessed by electrophoresis in agarose-formaldehyde gels. Equal amounts of RNA were subjected to reverse transcription to synthesize cDNAs (Reverse Transcription System, Promega Co).

PCR

PCR was carried out in a total reaction volume of 20 μ l containing 4 μ l cDNA. The 30 cycles of PCR amplification were preceded by a heat denaturing step at 95 °C for 5 min. Each cycle of PCR consisted of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The PCR was terminated with a prolonged extension step at 72 °C for 5 min. Primers were designed with Gene Runner Analysis software (Hastings Software Inc., Hastings on Hudson, NY,

USA). The nucleotide sequences for the primers were listed in Table 1. Products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide, and visualized under u.v. light.

Preparation of FQ-PCR standards

TR α 1, TR α 2, TR β 1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) target fragments were recovered and purified from 1.5% agarose gel using a PCR product purification kit (Beijing BioDev-Tech Co., Ltd, Beijing, China). The target fragments were cloned into PMD18-T vector (TIANGEN Biotech Co., Ltd, Beijing, China) respectively and the recombinants were transformed into competent DH5 α bacteria, followed by screening of positive clones. Recombinant plasmid-carrying bacteria suspension was sent to Shanghai Bioasia Co., Ltd for sequencing analysis. Meanwhile, recombinant plasmids were extracted and A260 values were measured to clear the concentration of each recombinant plasmid. The copy number of recombinant plasmids was converted as follows: copy number = mass (g) $\times$ 6.023 $\times$ 10²³ / 660 $\times$ base number. The solution of plasmids was diluted to 10⁸, 10⁷, 10⁶, 10⁵, 10⁴, and 10³ copy/ μ l respectively. Thus, FQ-PCR standards for TR α 1, TR α 2, TR β 1, and GAPDH were obtained. The standards were stored at -20 °C before use.

Plotting FQ-PCR standard curve

TaqMan probes (with the fluorescent substance 6-carboxy-fluorescein (FAM) at the 5'-terminal and the fluorescence quencher 6-carboxytetramethylrhodamine (TAMRA) at the 3'-terminal) were synthesized according to the sequences as listed in Table 1 (Shanghai Bioasia Co., Ltd, Shanghai, China). FQ-PCR amplification was carried out using the following cycle conditions: Stage 1 (denaturation), 95 °C for 5 min; Stage 2 (amplification), 40 cycles of 95 °C for 15 s, 63 °C for 40 s; Stage 3 (melting curve), 95 °C for 0 s, 45 °C for 15 s, 95 °C for 0 s; and Stage 4 (cooling), 40 °C for 30 s. FQ-PCR standards were amplified using Roche Lightcycler System for real-time PCR to determine the correlation of the standard curve.

Table 1 Nucleotide sequences for thyroid hormone receptor α 1 (TR α 1), TR α 2, TR β 1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and TaqMan probe

	Nucleotide sequences	Amplification site
Target		
	Sense 5'-TGCCCTTACTCACCCCTACA-3'	1897-1916
	Antisense 5'-AAGCCAAGCCAAGCTGTCCT-3'	2009-2028
TR α 1	Probe 5'-TTCAGGCCACAGTCTCTACTTCCC-3'	1960-1984
	Sense 5'-TGAGCAGCAGTTTGGTGAAG-3'	1211-1230
	Antisense 5'-GAATGGAGAATTCCGCTTCG-3'	1308-1327
TR α 2	Probe 5'-TTCAGCACCAGAGCCCGAAGAG-3'	1255-1276
	Sense 5'-AGCCAGCCACAGCACAGTGA-3'	4017-4036
	Antisense 5'-CGCCAGAAGACTGAAGCTTGC-3'	4116-4136
TR β 1	Probe 5'-ACGAACCAGGCTGTAGCAACCGTGG-3'	4068-4092
	Sense 5'-CCTGGAGAAACCTGCCAAGT-3'	1587-1605
	Antisense 5'-TGGGAGTTGCTGTTGAAGTC-3'	1696-1715
GAPDH	Probe 5'-CATCAAGAAGGTGGTGAAGCAGGC-3'	1614-1637

FQ-PCR detection of samples

Total RNA was extracted from myocardial samples. cDNAs were obtained by reverse transcription and then amplified along with the standards using Roche Lightcycler System. The amplification conditions were the same for both the sample cDNAs and the standard cDNAs. Thus, the copy number of TR α 1, TR α 2, TR β 1, and GAPDH mRNA per μ l was calculated for each of the samples. Following each FQ-PCR, a melting curve analysis was performed to ensure the specificity of FQ-PCR.

Pathologic study

Rat hearts from the CH group and the control group were washed with DEPC water to remove blood in the cardiac chambers and then stored in liquid nitrogen. Rat hearts were sectioned into a thickness of $\sim 10\ \mu$ m using a thermostatic freezing microtome CM1850 (Leica, Leica Microsystems GmbH, Wetzlar, Germany) at $-20\ ^\circ\text{C}$. The tissue was loaded onto glass slides, fixated in a mixture of 95 ml of 95% ethanol and 5 ml glacial acetic acid for 1 min, and washed with running water. The sections were subjected to routine hematoxylin and eosin (HE) staining, mounting with neutral resin, and photography using an Olympus image analyzer (Olympus, Tokyo, Japan).

Statistical analysis

Each experiment was repeated thrice. All data were expressed as means $\pm$ S.D. TR α 1, TR α 2, and TR β 1 mRNA levels were analyzed by independent samples *t*-test to determine whether the expression of mRNAs encoding TR receptors varied between normal and hyperthyroid groups or between normal and congenital hypothyroid groups. Levels of expression of each mRNA detected were normalized to GAPDH mRNA. $P < 0.05$ was considered to be significant.

Results

Hyperthyroid animals

Compared with the control group, the weight of rats from the hyperthyroid group increased more slowly, the heart rate (HR) was faster, and serum FT₃ and FT₄ levels were higher ($P < 0.01$). Hyperthyroid rats were irritable, they ate and drank significantly more, and their hair lacked luster. Macroscopic observations revealed obvious myocardial hypertrophy and cardiac chamber

narrowing in hyperthyroid rats (5/8), and heart weight was higher in the hyperthyroidism group than in the control group ($P < 0.01$; Tables 2 and 3).

Hypothyroid animals

The neonate mortality rate was higher in the hypothyroid group (30%) than in the control group. Hypothyroid rats grew slowly and were small with a short tail. They opened their eyes late. Their hair grew slowly and was sparse. They were weaned late. Their balancing capability was poor and they reacted to stimuli slowly. The body weights (BW) of P0d, P14d, P21d, and P45d hypothyroid rats were lower than those in the corresponding control group ($t = 3.97, 8.66, 8.91, 5.72, P < 0.01$). The FT₃ levels of P0d, P14d, and P21d hypothyroid rats were lower than those in the corresponding control group ($t = 2.84, 5.81, 5.20, P < 0.01$). The FT₄ levels of P0d, P14d, and P21d hypothyroid rats were lower than those in the corresponding control group ($t = 3.64, 6.98, 4.97, P < 0.01$; Table 4).

PCR results of TRs

Sequencing results indicated that the sequences of target genes in the recombinant plasmids were identical to those registered in the GenBank database (Fig. 1).

FQ-PCR standard curve

As shown (Fig. 2), the points standing for the concentration gradients were in a straight line, with a correlation coefficient of 1.00, which denoted a high reliability of the standard curve. The melting curve analysis displayed the single specific product peak appearing per test, which ensured the specificity of FQ-PCR. FQ-PCR standard curve was plotted with the logarithms of initial copy numbers of the standard preparations as abscissa and C_t values as ordinate. There was good linearity between C_t values and the logarithms of initial copy numbers of the standard preparations ($r = -1.00$).

Differential expression of mRNA for TR α 1, TR α 2, and TR β 1 between normal and hyperthyroid rats myocardium

Compared with the normal myocardium, TR α 1 mRNA expression was upregulated by 51% ($t = 9.17, P < 0.01$), TR α 2 mRNA expression was downregulated by 58% ($t = 10.02, P < 0.01$), and TR β 1 mRNA expression remained

Table 2 Body weight (BW) changes in control and hyperthyroid rats ($n = 8, \bar{X} \pm S, \text{g}$)

	0 w	3 w	BW changes
Group			
Control	175.64 $\pm$ 11.80	275.53 $\pm$ 35.47	99.89 $\pm$ 24.36
Hyperthyroid	173.17 $\pm$ 10.90	231.19 $\pm$ 10.01	58.01 $\pm$ 14.78*

The weight of rats from the hyperthyroid group increased more slowly than the control group. $t = 4.158$.
* $P < 0.01$ versus control group.

Table 3 Serum free tri-iodothyronine (FT₃) and free thyroxine (FT₄) levels, heart rate (HR), and heart weight (HW) in control and hyperthyroid rats ($n=8$, $\bar{X} \pm S$)

	Control	Hyperthyroid	<i>t</i>
FT ₃ (pmol/l)	1.01 ± 0.23	9.99 ± 2.22*	11.37
FT ₄ (pmol/l)	18.46 ± 4.54	62.99 ± 12.27*	9.63
HR (beats/min)	439.13 ± 25.71	527.75 ± 26.47*	6.79
HW (g)	0.95 ± 0.16	1.32 ± 0.16*	4.57

Serum FT₃ and FT₄ levels, heart rate, and heart weight were higher in the hyperthyroid group than in the control group. * $P < 0.01$ versus control group.

unchanged in hyperthyroid rat myocardium ($t=0.96$, $P > 0.05$; Fig. 3).

Differential expression of mRNA for TR α 1, TR α 2, and TR β 1 between normal and hypothyroid rats myocardium

TR α 1, TR α 2, and TR β 1 were expressed in normal and hypothyroid rat myocardium throughout the developmental process.

In normal rat myocardium, TR α 1 mRNA expression was the highest at birth and then decreased gradually to the adult levels. Compared with normal rat myocardium, TR α 1 mRNA expression was downregulated by 90% at P0d ($t=18.91$, $P < 0.01$), 25% at P21d ($t=3.94$, $P < 0.01$), and 41% at P45d ($t=5.88$, $P < 0.01$) in hypothyroid rat myocardium, with the expression peak appearing until 2 weeks postnatally. Hence, in hypothyroid rats, myocardial TR α 1 mRNA expression was generally downregulated and the expression peak appeared late (Fig. 4).

In normal rat myocardium, TR α 2 mRNA expression was the highest at birth and then decreased gradually to the adult levels. Compared with normal rat myocardium, TR α 2 mRNA expression was upregulated by 27% at P0d ($t=7.60$, $P < 0.01$), 71% at P14d ($t=20.94$, $P < 0.01$), 78% at P21d ($t=22.05$, $P < 0.01$), and 36% at P45d ($t=9.46$, $P < 0.01$) in hypothyroid rat myocardium, with the expression peak appearing until 2 weeks postnatally. Hence,

in hypothyroid rats, myocardial TR α 2 mRNA expression was generally upregulated and the expression peak appeared late (Fig. 5).

In normal rat myocardium, TR β 1 mRNA expression reached a peak at P14d and then decreased gradually to the adult levels. Compared with normal rat myocardium, TR β 1 mRNA expression was downregulated by 75% at P0d ($t=61.97$, $P < 0.01$), 64% at P14d ($t=9.44$, $P < 0.01$), 66% at P21d ($t=30.86$, $P < 0.01$), and 67% at P45d ($t=11.25$, $P < 0.01$) in hypothyroid rat myocardium. Hence, in hypothyroid rats, myocardial TR β 1 mRNA expression was generally downregulated and changed similarly with the control group (Fig. 6).

Pathologic study

Observations of HE-stained sections showed no morphological differences of myocardium between control P0d and hypothyroid P0d rats. In all P0d rats, myocardial pedomorphism was observed, i.e., myocardial cells contained a little sarcoplasm, but no myofibrils or transverse striations, and cell boundary was indistinct. In the control group, myocardial sarcoplasm became abundant and obvious myofibrils or transverse striations were observed at P14d, P21d, and P45d. In contrast, in hypothyroid group, myocardial cell maldevelopment was observed at 14 and 21 days postnatally, with a little sarcoplasm, a thin and long cell shape, and

Table 4 Body weight (BW), serum free tri-iodothyronine (FT₃) and free thyroxine (FT₄) levels in each group ($n=12$, $\bar{X} \pm S$)

Group	BW (g)	FT ₃ (pmol/l)	FT ₄ (pmol/l)
Control			
0d	6.80 ± 0.68	0.20 ± 0.08	3.98 ± 0.70
14d	35.11 ± 2.73	1.31 ± 0.29	92.18 ± 30.43
21d	61.19 ± 3.79	1.4 ± 0.32	53.20 ± 6.85
45d	169.55 ± 13.12	1.51 ± 0.28	73.41 ± 19.78
Hypothyroid			
0d	5.48 ± 0.53*	0.10 ± 0.05*	1.77 ± 0.25*
14d	24.04 ± 2.66*	0.55 ± 0.11*	4.69 ± 1.82*
21d	39.19 ± 4.55*	0.86 ± 0.26*	28.24 ± 4.46*
45d	124.26 ± 14.13*	1.42 ± 0.48	65.57 ± 6.31

The body weight, FT₃ and FT₄ of P0d, P14d, and P21d hypothyroid rats were lower than those in the P0d, P14d, and P21d control group respectively. * $P < 0.01$ versus control group.

Figure 1 PCR results of TRα1, TRα2, and TRβ1.

indistinct transverse striations. Moreover, Henle's fissures (spaces between myocardial cells) were widened and myocardial cells were deranged focally. At 45 days postnatally, in the hypothyroid group, myocardial transverse striations can be observed, but sarcoplasm was still not abundant and cells were thin and long, with widened Henle's fissures (Fig. 7).

Discussion

The heart is a major target organ for thyroid hormone (T₃) action, and marked changes occur in cardiac faction in patients with hypo- or hyperthyroidism. T₃ significantly influences cardiac contractile and electrophysiological function with increasing amounts of T₃ enhancing cardiac contractile function and accelerating HR. These effects are mediated by the binding of T₃ to specific nuclear TRs. TRs are members of a family of nuclear receptors that includes steroid, retinoic acid, and vitamin D receptors, all closely related to the v-erbA oncogene. TRs are encoded by two different genes: TRα on chromosome 17 and TRβ on chromosome 3. TRα and TRβ isoforms have a similar structure, characterized by an amino-terminal domain, a DNA-binding domain comprises two zinc finger motifs, and a carboxy-terminal ligand-binding domain. The TRα gene generates the TRα1 and TRα2 isoforms that are identical for

Figure 2 FQ-PCR standard curve.

Figure 3 FQ-PCR analysis of TRα1, TRα2, and TRβ1 mRNA expression differentiations between control and hyperthyroid rat myocardium ($\bar{X} \pm S$, $n=8$). The TRα1 mRNA expression was upregulated by 51%, TRα2 mRNA expression was downregulated by 58%, and TRβ1 mRNA expression remained unchanged in hyperthyroid rat myocardium. * $P<0.01$, * $P<0.05$ compared with the control group.

the first 370 amino acids but differ as a consequence of differential splicing at their C terminus: the last 40 specific residues of TRα1 are replaced by 122 amino acids encoded by the TRα2-specific exon 10, the last exon in the TRα locus. As a consequence of the C-terminal change, the TRα2 protein is unable to bind TH and no other ligand has been identified. TRα2 binds DNA weakly and only binds a subset of T₃-responsive sites. Furthermore, it dimerizes poorly with RXR and lacks the activating function domain 2 that interacts with coactivators. Both TRα1 and TRα2 mRNA are widely distributed. Alternative promoters or splicing of the amino-terminal region of the TRβ mRNA forms TRβ1 and TRβ2 in rat, both of which bind T₃. The TRβ2 mRNA is concentrated primarily in the anterior pituitary and the central nervous system of the rat, whereas the TRβ1 mRNA is widely distributed. Therefore, in our experiments,

Figure 4 FQ-PCR analysis of TRα1 mRNA expression differentiations between control and hypothyroid rat myocardium ($\bar{X} \pm S$, $n=5$). Compared with normal rat myocardium, TRα1 mRNA expression was downregulated by 90% at P0d, 25% at P21d, and 41% at P45d in hypothyroid rat myocardium, with the expression peak appearing until 2 weeks postnatally. * $P<0.01$ compared with the control group.

Figure 5 FQ-PCR analysis of TR α 2 mRNA expression differentiations between control and hypothyroid rat myocardium ($\bar{X} \pm S$, $n=5$). TR α 2 mRNA expression was upregulated by 27% at P0d, 71% at P14d, 78% at P21d, and 36% at P45d in hypothyroid rat myocardium, with the expression peak appearing until 2 weeks postnatally. * $P < 0.01$ compared with the control group.

we detected TR α 1, TR α 2, and TR β 1 mRNA alterations except β 2. In addition, TR α 1 and TR β 1 are thyroid hormone functional receptors except TR α 2 (Sap *et al.* 1986, Weinberger *et al.* 1986, Thompson *et al.* 1987, Lazar & Chin 1990).

In our experiment, the phenotype of the hyperthyroid rat has features of hyperthyroidism, including slowly increased BW, elevated HR, cardiac hypertrophy, and higher serum FT $_3$ and FT $_4$ levels. The phenotype of the hypothyroid rat also has features of CH, such as slow growth, late weaning, poor balancing capability, slower reaction to stimuli, and lower serum FT $_3$ and FT $_4$ levels. Thus, the animal models were made successfully.

Our results have shown that TR α 1 mRNA expression was upregulated, TR α 2 mRNA expression was downregulated, but TR β 1 mRNA expression remained unchanged in the hyperthyroid rat myocardium compared with the normal myocardium. This may be because: 1) TR α 1 is the major functional receptor in the myocardium; deficiency in TR isoforms caused discrete alterations in HR and ventricular

Figure 6 FQ-PCR analysis of TR β 1 mRNA expression differentiations between control and hypothyroid rat myocardium ($\bar{X} \pm S$, $n=5$). TR β 1 mRNA expression was downregulated by 75% at P0d, 64% at P14d, 66% at P21d, and 67% at P45d in hypothyroid rat myocardium. * $P < 0.01$ compared with the control group.

Figure 7 Observe the morphological differences of myocardium between control group and hypothyroid group rat. (A) Rat myocardium in P0d control group, HE staining, 100 $\times$. (B) Rat myocardium in P45d hypothyroid group (myocardial transverse striations can be observed, but sarcoplasm was still not abundant, with widened Henle's fissures.), HE staining, 400 $\times$. (C) Rat myocardium in P21d control group, HE staining, 200 $\times$. (D) Rat myocardium in P21d hypothyroid group (with widened Henle's fissures, myocardial cells were deranged focally), HE staining, 200 $\times$. (E) Rat myocardium in P21d control group, HE staining, 400 $\times$. (F) Rat myocardium in P21d hypothyroid group (with a little sarcoplasm and indistinct transverse striations), HE staining, 400 $\times$.

function. The TR α 1 $^{-/-}$ mice exhibited lower HR, lower body temperature, and slower ventricular repolarization (prolonged P-Q, QRS, and Q-T durations in the ECG) as compared with normal mice. The TR β 1 $^{-/-}$ mice exhibited severely impaired hearing, normal HR as well as normal contractile function of papillary muscle (Forrest *et al.* 1996a,b, Rüsch *et al.* 1998, Wikström *et al.* 1998, Göthe *et al.* 1999, Johansson *et al.* 1999). Comparison of the cardiac phenotype between TR α and TR β KO mice clearly indicates that TR α had predominant contractile and electrophysiological function in the heart. Furthermore, TR α 1 mRNA was present at a 3:1 ratio to TR β mRNA in the ventricle of the heart (Swanson *et al.* 2003). These findings indicated that the cardiac phenotype regulated by T $_3$ was primarily mediated by the more predominant TR α in the heart. The lack of TR α cannot be compensated by TR β in the heart, but the lack of TR β can be compensated by TR α in the heart (Gloss *et al.* 2001). The TR α 1 exerts a predominant effect on cardiac electrophysiological functions. Thus, at high thyroid hormone level, the expression of TR α 1, the major functional

receptor of thyroid hormones in the myocardium was upregulated in a compensatory manner. 2) The TR α 2 has been suggested to exert a suppressive function on other TRs. Suggested mechanisms for suppression include competition for binding to thyroid hormone response elements (TREs) on target genes, formation of inactive heterodimers, or sequestration (Rentoumis *et al.* 1990, Katz *et al.* 1992, Katz & Lazar 1993, Liu *et al.* 1995, Tagami *et al.* 1998). Transient transfection of the cDNA coding for TR α 2 has been shown to block the effect of a co-transfected α 1 or β 1 cDNA in facilitating T₃ regulation of a third co-transfected reporter gene (Koenig *et al.* 1989, Strait *et al.* 1990). This blockade has been ascribed to an ability of the TR α 2 protein to compete with α 1 and β 1 for the TRE situated in the 5'-flanking region of the reporter gene. In addition, selective ablation of TR α 2 resulted in an inevitable, concomitant overexpression of TR α 1 (Saltó *et al.* 2001). So down-regulation of TR α 2 mRNA expression attenuates the inhibition of TR α 1 by TR α 2 and leads to upregulation of TR α 1 expression. Upregulated TR α 1 can lead to heart lesions through modulating the expression of T₃-regulated genes. MHC has a high ATPase activity and is closely related to fast myocardial contraction. The TR α 1 expression upregulation may upregulate the expression of MHC α mRNA and enhance myocardial inotropy (Kinugawa *et al.* 2001). A long-term hyperkinetic state of myocardium causes myocardial strain to progress from the compensated to the decompensated stage. Na⁺/H⁺ exchanger-1 (NHE1) is a plasma membrane protein that removes an intracellular proton, exchanging it with extracellular sodium. By doing so, the NHE1 raises intracellular pH. The TR α 1 can activate the NHE1 exchanger promoter (Li *et al.* 2002). The TR α 1 expression upregulation may lead to NHE1 overactivation, thus causes acidosis in myocardial cells and progressive degeneration and even necrosis of myocardial cells. These changes are consistent with myocardial pathological changes in patients with chronic hyperthyroid heart disease.

Thyroid hormone signaling is critical for proper heart development and function. T₃ can increase expressions of myocardium-specific TH-responsive genes. Myocardium-specific genes comprise a list of proteins responsible for cardiomyocyte function: MHC, SERCA2, α - and β -adrenergic receptors, several plasma membrane ion transporters, such as Na⁺/Ca²⁺ exchanger, NHE, and a variety of ion channels, including HCN2, HCN4, Kv1.5, Kv4.2, and Kv4.3. They can be regulated at both the transcriptional and post-transcriptional levels by T₃ binding with specific nuclear TRs, thus coordinating the electrochemical and mechanical responses of the myocardium (Ma *et al.* 2003, Kahaly & Dillmann 2005). TH-responsive gene promoters are selectively regulated by the various TR isoforms. For example, TR α 1 can activate the α -MHC, HCN2, and HCN4 promoter and TR β 1 can activate the HCN4 promoter and repress the β -MHC promoter (Gloss *et al.* 2001). Our experiment showed that in hypothyroid rats, TR α 1 mRNA expression was generally downregulated and the expression peak appeared late, and

TR β 1 mRNA expression was generally downregulated. Downregulated TR α 1 and TR β 1 mRNA expression will lead to the decreased expression of HCN2, HCN4, MHC α , and SERCA2. HCN2 and HCN4 have been identified as components of an ion channel that constitutes the I_f current, which contributes to HR generation. Markedly decreased expression of HCN2 and HCN4 occurs in TR α KO mice and euthyroid TR β PV with a decreased HR (Gloss *et al.* 2001). MHC α mRNA code protein that is linked to force generation. SERCA2 mRNA code protein that is linked to calcium lowering during diastole, so decreased levels of MHC α mRNA and SERCA2 mRNA may provide the cause for slowed force development and prolonged relaxation time. Thus, the abnormal expressions of myocardium-specific genes caused myocardial structural developmental abnormalities and serious heart dysfunction. These changes are consistent with myocardium pathological changes in patients with chronic hypothyroid heart disease.

Abnormal expressions of TR subtypes in hypothyroid adult rat myocardium have been reported previously, but the results were inconsistent, possibly due to different preparation methods of animal models and different detection methods adopted by those laboratories. In the present study, we established congenital hypothyroid rats and monitored the influence of CH on myocardial TR subtypes expressions from birth through adulthood. We not only reported the changes of myocardial TR expressions in normal rats throughout the developmental process, but also demonstrated delayed peaks and down-regulation of expressions of functional receptors of thyroid hormone in hypothyroid rats. In addition, the study confirmed that CH caused myocardial maldevelopment (less sarcoplasm in the thin and long cell, transverse striations development delay and widened Henle's fissures) may be closely associated with abnormal TR expressions. TR α 1 and TR β 1 are functional receptors of thyroid hormones in the myocardium, and downregulated TR α 1 and TR β 1 expression may lead to abnormal expression of multiple genes regulated by thyroid hormones, thus resulting in a series of electrophysiological abnormalities of the heart and myocardial functional injury. In addition, thyroid hormones are crucial for myocardial development and they may promote myocardial cell survival. Lack of thyroid hormones in the embryonic stage may induce myocardial cell apoptosis and downregulate the mRNA expressions of functional thyroid hormones receptors (Alisi *et al.* 2005, Verga *et al.* 2006).

At an early stage of the experiment, we wanted to establish a neonatal hyperthyroidism model to monitor the influences of hyperthyroid on myocardial TR expressions from birth through adulthood, but the mortality of pregnant and neonate rat were so high that adequate samples cannot be obtained. Considering hyperthyroidism is more common in adults than in children, we had to alter the experiment design. We had to give up the neonate hyperthyroidism model and establish an adult hyperthyroidism model to detect the effect of thyroid hormone on the adult hyperthyroid rat myocardium. In the future, we

plan to study TR's differential expressions in hyperthyroid developmental myocardium on the basis of this article.

In summary, the present study demonstrated that abnormal thyroid hormone concentrations caused abnormal TR mRNA expressions in the myocardium. During hyperthyroidism, high thyroid hormone level may influence the transcription level of TR and lead to faster HR and a hyperkinetic state of the heart by influencing the expressions of thyroid hormone-regulated genes. Hence, a long-term exposure to high thyroid hormone level will lead to heart failure. During CH, TR mRNA expression peak appeared late and abnormal expression changes of TRs occurred in the myocardium, which resulted in maldevelopment of myocardial cells and conduction bundles, irreversible damage to myocardial structure and function, and ultimately hypothyroid heart disease. Therefore, in the presence of thyroid dysfunction, abnormal expressions of TR subtypes in the myocardium are closely associated with the pathogenesis of heart lesions. The present study not only deepened the understanding of pathogenesis of thyroid dysfunction caused heart lesions, but also may guide gene therapy and the selective application of various TR subtype antagonists for such heart lesions.

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