Regulation of Thyroid Hormone Receptor Isoforms in Physiological and Pathological Cardiac Hypertrophy

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Abstract—Physiological and pathological cardiac hypertrophy have directionally opposite changes in transcription of thyroid hormone (TH)-responsive genes, including α- and β-myosin heavy chain (MyHC) and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), and TH treatment can reverse molecular and functional abnormalities in pathological hypertrophy, such as pressure overload. These findings suggest relative hypothyroidism in pathological hypertrophy, but serum levels of TH are usually normal. We studied the regulation of TH receptors (TRs) β1, α1, and α2 in pathological and physiological rat cardiac hypertrophy models with hypothyroid- and hyperthyroid-like changes in the TH target genes, α- and β-MyHC and SERCA. All 3 TR subtypes in myocytes were downregulated in 2 hypertrophy models with a hypothyroid-like mRNA phenotype, phenylephrine in culture and pressure overload in vivo. Myocyte TRβ1 was upregulated in models with a hyperthyroid-like phenotype, TH (triiodothyronine, T3), in culture and exercise in vivo. In myocyte culture, TR overexpression, or excess T3, reversed the effects of phenylephrine on TH-responsive mRNAs and promoters. In addition, TR cotransfection and treatment with the TRβ1-selective agonist GC-1 suggested different functional coupling of the TR isoforms, TRβ1 to transcription of β-MyHC, SERCA, and TRβ1, and TRα1 to α-MyHC transcription and increased myocyte size. We conclude that TR isoforms have distinct regulation and function in rat cardiac myocytes. Changes in myocyte TR levels can explain in part the characteristic molecular phenotypes in physiological and pathological cardiac hypertrophy. (Circ Res. 2001;89:591-598.)

Key Words: thyroid hormone receptor ■ physiological and pathological hypertrophy ■ α-3-adrenergic receptor ■ cardiac myocyte ■ rat

Cardiac hypertrophy is sometimes considered a single process that leads invariably to myocardial dysfunction (pathological hypertrophy). However, physiological hypertrophy exists in which cardiac function is maintained or enhanced, including normal cardiac development, exercise training, and thyroid hormone (TH) treatment. Exercise and TH can reverse molecular and functional abnormalities in pathological hypertrophy without decreasing ventricular mass, indicating that physiological and pathological hypertrophy are qualitatively distinct processes.1-6 TH-responsive genes in cardiac muscle include α-myosin heavy chain (MyHC) and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), which are induced by TH, and β-MyHC, which is repressed.7,8 An intriguing observation is that pathological hypertrophy is characterized by hypothyroid-like changes in these target genes, with decreases in α-MyHC and SERCA and increases in β-MyHC, a molecular phenotype also called the fetal program.9 The fact that TH treatment can reverse these genetic changes in some models of pathological hypertrophy is additional evidence for a hypothyroid state, but TH blood levels are usually normal.3 Conversely, physiological hypertrophy caused by exercise is characterized by hyperthyroid-like changes in TH target genes, yet again blood TH levels are normal.10 Only in cardiac hypertrophy during normal postnatal development are changes in TH-responsive genes attributable clearly to altered TH levels.11,12

Transcription of TH target genes is mediated by nuclear TH receptors (TRs), with 2 genes, α and β, encoding at least 4 major TRs, β1, β2, α1, and α2, as well as minor variants, including TRβ3.15 TRs β1, α1, and α2 are expressed widely. TRβ2 is present mainly in pituitary cells but might be a small fraction of T3-binding TRs in heart, as found in one study.16 TRα2 binds thyroid response elements (TREs) on DNA but does not bind 3,3’,5-triiodo-L-thyronine (T3), the
active form of TH, and can function as a dominant negative.11,13 TRs β1 and α1 bind T3 with similar affinity but exhibit subtle differences in binding to TREs and cofactors and in forming homodimers and heterodimers with retinoid X receptors.13,18–20 Consistent with these biochemical differences, mouse TR knockouts reveal nonredundant TR functions in many tissues.19–21 In the heart, however, TR knockouts suggest that the transcription effects of TH are mediated largely by TRα1.17,22 TR functions have not been evaluated systematically in isolated myocytes.

In this study, we tested the hypothesis that hypothyroid- and hyperthyroid-like transcription changes in pathological and physiological hypertrophy are caused by altered TR levels in heart and myocytes. Recent studies in the aging rat and in human end-stage heart failure reveal changes in myocardial TR levels that correlate with changes in TH target gene transcription.23,24 However, possible changes in TR levels in hypertrophy have not been examined, and TR functions on target promoters in myocytes have not been compared. We measured TRs in rat hypertrophy models in culture and in vivo and tested TR functions in culture using transfection and treatment with T3 or a TRβ1-selective agonist.

**Materials and Methods**

**Cell Culture, Hypertrophy, and Transfection**

Ventricular myocytes from 1-day-old rats were plated at low density in MEM with 5% calf serum and studied in serum-free MEM exactly as in Deng et al.25 Cardiac nonmyocytes in preplates were expanded to near confluence in MEM with 5% serum.26 Cultures were treated with phenylephrine (PE) (Sigma No. P6126), T3 (Sigma No. T5516), GC-1 (gift from G. Chieffini and T.S. Scanlan, Department of Pharmaceutical Chemistry and Cellular and Molecular Pharmacology, University of California, San Francisco, Calif),18 or their vehicles (ascorbic acid for PE, NaOH for T3, and DMSO for GC-1). Myocyte hypertrophy was quantified by content of radiolabeled protein (RLP) after continuous incubation with 14C- or 3H-phenylalanine. Myocytes were doubly transfected using calcium phosphate coprecipitation27 with expression plasmids for the human α1-adrenergic receptor (AR) hypertrophic agonist PE (20 μmol/L) had opposite effects on these mRNAs, increasing β-MHC and decreasing SERCA and α-MHC. Prazosin (2 μmol/L) inhibited all PE effects, confirming α1-AR dependence (n = 3 cultures, data not shown).

Under our basal, serum-free culture conditions, medium T3 levels averaged about 0.1 nmol/L over the 3-day time course of these experiments, presumably reflecting myocyte release of stored T3.33–35 Interestingly, when 1000-fold excess T3 (100 nmol/L) was added to PE-treated myocytes, all PE effects on TH-responsive transcripts were reversed, with significant increases in α-MHC and SERCA and decreases in β-MHC (Table 1). The antagonistic relationship between α1-AR and T3 signaling was not explained by a lesser degree of hypertrophy, because hypertrophy was even greater with PE plus T3 (Table 1).

In intact rat hypertrophy models, voluntary running exercise stimulated hypertrophy with an mRNA profile similar to T3 in culture (Table 1). Conversely, pressure overload by ascending aortic constriction caused hypertrophy with changes in TH target mRNAs opposite to exercise and similar to PE in culture (Table 1). Thus, in agreement with previous studies (discussed in the Introduction), 2 hypertrophy models had signature mRNA changes of heightened thyroid signaling, T3 in culture and exercise in vivo. In contrast, 2 other models had a directionally opposite pattern of hypothyroid-like signaling, PE in culture and pressure overload in vivo. T3 and T4 blood levels in the intact rat models were similar among all 4 groups, with total T4 ~40 nmol/L (3 to 3.2 μg/dL) and total T3 ~1 nmol/L (52 to 55 ng/dL) (n = 6 to 13, P = NS), as found by others.3,10 Thus, mRNA changes were not explained by changes in circulating TH concentration.

**Results**

**TH-Responsive mRNAs in Hypertrophy Models in Culture and In Vivo**

We first confirmed regulation of TH target mRNAs in cardiac hypertrophy models in culture and in vivo (Table 1). In cultured neonatal rat myocytes, TH (T3 100 nmol/L) caused hypertrophy, increased α-MHC andSERCA mRNA levels, and repressed β-MHC to 5% of control. The α1-adrenergic receptor (AR) hypertrophic agonist PE (20 μmol/L) had opposite effects on these mRNAs, increasing β-MHC and decreasing SERCA and α-MHC. Prazosin (2 μmol/L) inhibited all PE effects, confirming α1-AR dependence (n = 3 cultures, data not shown).
α2, α1, and β1, with a molar ratio of ≈7:3:1. TRβ2 mRNA was not found, using a probe validated in rat pituitary GC cells (not shown), and TRβ3 was not tested. Treatment with T3 under conditions that stimulated hypertrophy (Table 1) caused an increase in TRβ1 mRNA, with a maximum ≈2-fold and an EC50 ≈3 nmol/L, but had no effect on TRα2 or α1 (Figure 1A). T3 induction of TRβ1 in myocytes was significant as early as 12 hours and maximum at 48 hours (data not shown). Thus TRβ1 itself was a TH-responsive gene in myocytes and was increased in T3-stimulated hypertrophy.

PE-induced hypertrophy in cultured myocytes had an effect different from T3 and reduced the levels of all 3 TR isoform mRNAs to 50% to 75% of control (Figure 1B). The EC50 for TR repression was about 3 μmol/L (Figure 1B), similar to the EC50 for PE-induced hypertrophy, and the PE effect was detected as early as 6 hours (data not shown). PE is an α1-AR agonist with weak β-AR activity, and specific α1-AR repression of TRs was confirmed in separate experiments with norepinephrine, isoproterenol, prazosin, and timolol (all 2 μmol/L, n = 3 to 4 cultures for each, data not shown). Interestingly, and similar to the other TH target mRNAs (Table 1), addition of T3 reversed PE repression of TRβ1, but TRs α1 and α2 were not changed (Figure 1B).

To test if TR mRNA levels reflected TR protein, we measured endogenous TRs in cultured myocytes by Western blot. None of the commercial antibodies from Santa Cruz or Affinity BioReagent gave specific signals for TRs α1 and α2 (not shown), but TRβ1 protein was detected in nuclear extracts (Figure 1). PE reduced TRβ1 protein significantly, whereas T3 increased TRβ1 and reversed TRβ1 repression caused by PE (Figures 1A and 1B). Thus TRβ1 mRNA and protein changes were concordant.

Cultured cardiac nonmyocytes also expressed mRNAs for all 3 of the TRs found in myocytes, α1, α2, and β1, but levels were not altered by PE 20 μmol/L or T3 100 nmol/L (n = 3 cultures, data not shown). No response to PE can be explained by the absence of α1-ARs in nonmyocytes. No response to T3 is compatible with a requirement for cell-specific factors such as MEF-2 in myocyte TR signaling.

## TR mRNA Levels in Hypertrophy Models In Vivo

Control adult male rat hearts contained mRNAs for TRs α2, α1, and β1 at equal molar ratios (≈1:1:1). Physiological hypertrophy stimulated by voluntary running exercise mimicked T3 in culture, with an increase in TRβ1 mRNA and no change in TRs α1 and α2 (Figure 2). Pathological hypertrophy stimulated by ascending aortic constriction was similar to PE in culture, with decreases in all 3 TR isoform mRNAs (Figure 2), detectable as early as 1 week after banding (not shown). Thus, in distinct hypertrophy models in vivo as in culture, TR isoform levels and TH-responsive transcript levels were related directly (Figure 2 and Table 1).

## Cotransfection of TR Isoforms and TH-Responsive Promoters

To test directly whether changes in TR levels could alter TH-responsive transcription, we cotransfected cultured myo-
cytes with expression plasmids for the human TR isoforms and reporter plasmids for the TH-responsive genes (Figure 3). The Western blot in Figure 3A shows that human TRβ1 was detected in nuclear extracts from TRβ1-transfected myocytes, with overflow of transfected TRβ1 into the cytosolic fraction, suggesting saturating levels. Similarly, an antibody recognizing both human and rat TRβ1 indicated that total TRβ1 nuclear levels in transfected cultures were about 2-fold over endogenous (Figure 3A). With ~5% of myocytes transfected, this result implied about 40-fold overexpression of TRβ1.

We used this system to test the effects of increasing TR isoform levels on α-MyHC, β-MyHC, and SERCA promoters. In vehicle-treated control myocytes, overexpression of TRα1 activated the α-MyHC promoter, and TRβ1 repressed β-MyHC (Figure 3B). TRα2 had no effect. Induction of α-MyHC indicated that TRα1 was expressed, even though Western blots were unsatisfactory for TRα protein levels.

In PE-treated myocytes transfectected with empty vector, the β-MyHC promoter was activated, and α-MyHC and SERCA promoters were repressed (Figure 3C), similar to the PE effects on endogenous mRNAs (Table 1). Increasing the levels of TRα1 by transfection reversed PE repression of α-MyHC, whereas TRβ1 overexpression significantly op-

posed the effects of PE on both β-MyHC and SERCA. Interestingly, forced expression of TRα2, the non-T3-binding TR, slightly but significantly enhanced PE repression of α-MyHC and SERCA (Figure 3C).

In T3-treated myocytes transfected with empty vector, the β-MyHC promoter was repressed and α-MyHC and SERCA were activated (Figure 3D), as expected from T3-induced mRNA changes (Table 1). Overexpression of TRβ1 in T3-treated myocytes significantly enhanced induction of the SERCA promoter, but TRs α1 and α2 had no effects over T3 alone (Figure 3D).

Taken together, these cotransfection results showed that increasing the levels of T3-binding TRs altered TH-responsive transcription and antagonized the effects of PE on TH-responsive genes. In addition, the results suggested different functions of TRs in myocytes, regulation of α-MyHC by TRα1, and regulation of β-MyHC and SERCA by TRβ1. The repressive effect of TRα2 only in PE-treated cells...
A. Western blot

Figure 3. TR transfection in cultured myocytes. Cultured myocytes were cotransfected with CAT reporter plasmids (5 μg) and CMV-driven human TR expression plasmids or equal amounts of empty CMV vector (2 to 5 μg). SV40-driven secreted alkaline phosphatase (1 μg, Clontech) was included to control for transfection efficiency. A, Western blots used TRβ1-specific antibodies from Santa Cruz (human-specific J51/SC-737 and rat- and human-reactive J52/SC-738) with myocyte nuclear and cytosolic extracts after 72 hours of transfection (left and middle). Rat and human TRβ1 proteins from in vitro transcription/translation were used as standards (~55-kD) (right). B through D, Myocytes were treated for 72 hours after transfection with vehicle (B); PE 20 μmol/L (C); or T3 10 nmol/L (D). Values are from 5 cultures.

Discussion

The main new finding in this study was the direct relationship between TR levels and transcription of TH-responsive genes in different types of hypertrophy. All TRs were downregulated in pressure overload, a model of pathological hypertrophy with a hypothyroid-like molecular phenotype. Conversely, TRβ1 was upregulated in the exercise model of physiological hypertrophy, where TH-responsive transcription was enhanced. These results, together with the TR overexpression experiments in cultured myocytes, suggest that the distinct molecular signatures in different types of hypertrophy can be caused by altered TR levels. Thus the fetal program in pathological hypertrophy can be viewed as the result of cellular hypothyroidism, caused by reduced TR levels, whereas physiological hypertrophy can be seen as a state of relative cellular hypothyroidism, attributable to increased TRβ1. The results also indicate that TR isoforms have different transcription functions in rat myocytes and raise the possibility of additional alteration of TH signaling in hypertrophy.

We detected 3 TRs in rat myocytes, β1, α1, and α2, and all were reduced by pressure overload in vivo and α1-AR signaling in culture (Figures 1 and 2), the models with hypothyroid-like transcription changes (Table 1). Reversal of hypothyroid signaling when TRs β1 and α1 were overexpressed in the PE culture model supported the causal significance of TR downregulation (Figure 3C). Downregulation of dominant-negative TRα2 in both models would have been expected to partly counteract the hypothyroid effects of decreased TRs β1 and α1, because overexpressed TRα2 had hypo- thyroid effects when TRs β1 and α1 were reduced by PE (Figure 3C). Nevertheless, global TR downregulation in both

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<td>mRNA level and promoter activity</td>
<td>β-MyHC</td>
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<tr>
<td>SERCA</td>
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<td>TRβ1</td>
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<td>α-MyHC</td>
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EC50s were estimated from the dose-response curves in Figures 1A and 4A through 4C. The curve for TRβ1 mRNA induction by GC-1 is not shown, and TRβ1 promoter activity was not tested. GC-1 is at least 10-fold less potent than T3 in stimulation of α-MyHC and RLP; the true EC50s for GC-1 induction of α-MyHC and RLP might be even higher, because the responses are not quite saturated at the highest doses studied (Figures 4B and 4C).
models of pathological hypertrophy was associated with reduced transcription of TH target genes.

Abnormal TR levels are also seen at a terminal stage of pathological hypertrophy, severe human heart failure. In human heart failure, TRα1 is decreased and TRα2 is increased, probably because of altered splicing of the TRα gene, and the increase in TRα2 correlates with reduced α-MyHC transcription. In our rat models of PE treatment and pressure overload, the global decrease in all TR mRNAs could be explained by reduced TR gene transcription or increased TR mRNA degradation. Thus the specific mechanism for altered TR levels might differ among species or models, but the human and rat studies both suggest that TR downregulation is a mechanism for hypothyroid-like signaling in pathological hypertrophy. This relationship was additionally strengthened by our converse results in physiological models.

TR upregulation was seen in exercise, a model of physiological hypertrophy with normal or enhanced contractile function. Voluntary running exercise caused an increase in TRβ1 but no change in TRα (Figure 2). T3 in culture also caused selective induction of TRβ1 (Figure 1) and similarly induces a physiological hypertrophy in vivo. Our finding that the TRβ1 gene is TH-responsive in myocytes but the TRα gene is not agrees with some studies in the intact rat, but not with others, and can be explained by positive TREs in the TRβ1 promoter that are absent in TRα. Furthermore, the TRβ1 gene seemed to be activated by TRβ1 itself, because T3 and the TRβ1-selective agonist GC-1 induced TRβ1 with similar potency (Table 2). Thus, exercise-induced increases in TRβ1 levels should be partly self-sustaining, and the increase in TRβ1 would augment transcription of downstream TH target genes on the basis of our transfection studies (Figure 3).

TRβ1 in myocytes differed from TRα1 in induction by T3 and also in function. In this study, we made the first systematic comparison of TR function in isolated myocytes and found by 2 complimentary approaches that TRβ1 induced SERCA and TRβ1 itself and repressed β-MyHC, whereas TRα1 increased α-MyHC and protein content (Figures 3 and 4 and Table 2). Notably, the same pattern of TR function was seen with TR cotransfection and with GC-1, which has 10-fold selectivity for TRβ1. Distinct TR isoform effects on different promoters might be explained by isoform-specific interactions with cofactors or differing affinities for TREs (see the Introduction). Interestingly, target gene–specific differences were evident even for a single TR isoform in that TRβ1 more potently repressed β-MyHC than it activated SERCA and activated SERCA more potently than TRβ1 itself (Table 2). Also intriguing was the correlation of TRα signaling with increased total protein content, an index of overall hypertrophy (Figure 4C and Table 2).
protein content requires increased translation as well as transcription, thus raising the question of nongenomic effects of T3 via TRα1.

Previous results from rat and human models are consistent with the distinct transcription functions of TR isoforms we show in this study. In the aging rat, increased β-MyHC transcription correlates with decreased TRβ1 levels and reduced α-MyHC correlates with reduced TRα1.23 A correlation between levels of TRα1 and α-MyHC is also seen in the failing human heart.24 A preferential effect of GC-1 on myocardial SERCA and β-MyHC is not seen in the rat in vivo, but this might be because of lower bioavailability of GC-C1 in cardiac tissue.35 On the other hand, the mouse might be different from rat and human, because knockout studies suggest that most cardiac effects of T3 can be accounted for by TRα1.17,22 Whether both TRs are required for full TH effects in heart is one key consideration in potential therapeuthic uses of TH signaling,8 and additional study is needed.

Finally, the question arises of whether other mechanisms alter TH signaling in hypertrophy in addition to changes in TR levels. Pertinent is the effect of T3 in the culture model, where a 1000-fold increase in medium T3 (from 0.1 to 100 nmol/L) reversed PE antagonism of all TH-responsive genes, whereas a 1000-fold increase in medium T3 (from 0.1 to 100 nmol/L) reversed PE antagonism of all TH-responsive genes, including the MyHCs, SERCA, and TRβ1, despite PE reduction of TR levels (Table 1 and Figure 1). Thus, excess T3 in culture could counteract the hypothypoxy effects of reduced TRs. In vivo as well, high doses of T3 reverse hypothypoxy transcription in pressure overload models5,33 despite the reduced TR levels we show in this study (Figure 2). The reversal effect of excess T3 can be explained partly by induction of TRβ1 and downstream changes in other TH-responsive genes, but the T3 effect also suggests that cellular T3 levels might be limiting in some cases. Thus it is possible that myocyte T3 metabolism35–38 is changed in hypertrophy, with increases in cellular T3 with exercise and decreases with pressure overload and PE, and this requires additional study.

In summary, changes in TR levels can explain at least in part the characteristic hypothypoxy- and hyperthyroid-like transcription in different types of hypertrophy. TH signaling is impaired in pathological hypertrophy and enhanced in physiological hypertrophy.

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Expanded Methods: Thyroid Hormone Receptors in Hypertrophy, Kinugawa et al.

RNase protection assays for TRs, MyHCs, and SERCA.

Probes for TR RNase protection assays

A. TRα probe

The Figure depicts the riboprobes used in RNase protection assay for TR mRNAs.

Similar shading within TR α or β isoforms indicates identical mRNA sequence. PF is the mRNA
fragment protected by the probe. The corresponding TR proteins are shown, with the DNA-binding domain (DBD) and ligand-binding domain (LBD) indicated. The TRα probe (Panel A) contained a PstI-XbaI fragment of rat TRα1 cDNA and protected a fragment of 538-bp for TRα1 (1279-1816 nucleotides of GenBank Accession number M18028) and 159-bp for TRα2 (1471-1629 nucleotides of M31174). The TRβ1-specific probe (Panel B) contained the fragment from the 5’-untranslated region to an XbaI site and protected 323-bp (227-555 nucleotides of J03933). The TRβ2-specific probe (Panel C) contained the fragment from the 5’-untranslated region to an XbaI site and protected 521-bp (1-521 nucleotides of M25071). All plasmids for riboprobes were sequenced, and linealized at the 3’-end of the antisense strand with appropriate restriction enzymes. TR probes were made with a specific activity of ~9x10⁸ cpm/µg, and hybridized with 10 µg total RNA. After incubation at 42°C overnight, the RNA-probe mix was digested with RNase A/T1 (1:100, RPAII, Ambion) at 37°C for 30 min, and protected fragments were separated on a 6% denaturing polyacrylamide gel. In each gel lane, all TR isoforms, and GAPDH as an internal control, were quantified by densitometry and corrected for background. TR signals were normalized to GAPDH.

The StyII-digested rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe (pTRI-GAPDH-Rat, Ambion) protected a 134-bp fragment (complimentary to nucleotides 369-503 of X02231). The GAPDH probe (specific activity ~2x10⁸ cpm/µg for experiments with MyHC and SERCA, ~0.5x10⁸ cpm/µg for experiments with TRs) was mixed in each sample as an internal control.

The probe specific for the rat SERCA2 gene protected a 333-bp fragment of SERCA2a (3014-3347 nucleotides of X15635). The probe for rat MyHC was complimentary to the 3’-end of β-MyHC mRNA; it protected a 300-bp fragment (5626-5925 nucleotides of X15939), but also hybridized to α-MyHC, and protected a 175-bp fragment (5656-5830 nucleotides of X15938). Riboprobes were labeled with [α-³²P]UTP using Maxiscript kit (Ambion). The MyHC and SERCA probes had a specific activity of ~0.5x10⁸ and ~1.0x10⁸ cpm/µg, respectively, and were
used with 3 µg total RNA. All probe mixes were run on the same gel with RNA from the in vivo experiments.