Expression of thyroid hormone receptor isoforms in rodents

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CHAPTER 7

Relationship Between TR Isoform Protein Expression and Target Gene Expression in Congestive Heart Failure

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Abstract

Cardiac hypertrophy develops in response to a variety of pathological stimuli, and may eventually progress from a compensatory state into heart failure when the chronic overload is sufficiently high. Hypertrophy and congestive heart failure are accompanied by characteristic changes in the expression of numerous cardiac genes. Expression of some of these genes is known to be regulated by thyroid hormone (TH). The genomic actions of TH require the binding of T3 to TH receptors (TR).

Because of the similarities in the pattern of gene expression in the diseased heart and the hypothyroid state, a role for TH in the development of cardiac hypertrophy or heart failure is possible. In the present study we investigated the time course and relationship between systemic TH status and cardiac TR isoform expression during the development of congestive heart failure (CHF). We determined the protein levels of the TR isoforms and the mRNA level of the MHC isoforms and of SERCA2a in right and left ventricles of rat hearts in which right ventricular pressure overload was induced by a single injection of monocrotaline (MCT). The pyrroloidineline alkaloid monocrotaline produces chronic pulmonary hypertension followed by the development of RV hypertrophy.

We show that there is a ventricle specific change in TR isoform expression as a result of the development of right ventricular hypertrophy eventually leading to heart failure. These changes in isoform expression are reflected in the expression of TH target genes and lead us to conclude that there appears to be, at least in our model, no clear connection between a particular target gene and a particular TR isoform.

Introduction

Cardiac hypertrophy develops in response to a variety of pathological stimuli, and may eventually progress from a compensatory state into heart failure when the chronic overload is sufficiently high. Hypertrophy and congestive heart failure are accompanied by characteristic changes in the expression of numerous cardiac genes, resulting in an impairment of Ca2+ handling and contractility. For instance, changes in the expression of the sarcoplasmic reticulum Ca2+-ATPase 2a (SERCA2a) and its regulatory molecule phospholamban (PLB) as well as of the myosin heavy chain (MHC) isoforms MHCα and MHCβ are thought to be critical for the systolic and diastolic dysfunction seen in heart failure. Expression of these genes is known to be regulated by thyroid hormone (TH) (3,4), as are a number of other cardiac genes implicated in heart failure, such as β1-adrenergic receptors, Gi-protein subunits, voltage-gated potassium channels and the Na+/Ca2+ exchanger (5,6). Because of the similarities in the pattern of gene expression in the diseased
heart and the hypothyroid state, a role for TH in the development of cardiac hypertrophy or heart failure has been suggested (7-9).

The genomic actions of TH require the binding of T3 to TH receptors (TR) associated with TH response elements in the promoters of TH responsive genes. Cellular uptake of T3 (10) as well as the abundance of TRs are therefore important parameters in cardiac gene regulation, and changes in cardiac TR expression have indeed been found in human and rat studies of heart failure (7,8). After binding of thyroid hormone to its receptor, the TR is activated followed by either activation or repression of their target genes. TRs are encoded by two different gene loci, TRα and TRβ. TRβ locus encodes TRβ1, TRβ2, TRβ3 which are all generated via alternative splicing and different promoter usage (11,12). The TRα gene encodes for TRα1 (able to bind thyroid hormone) with the splicing variant TRα2 and the truncated products (13-15).

Previous studies indicate that TR isoform mRNA or protein expression is differentially regulated in rat heart by thyroid hormone. Hypothyroidism results in a up-regulation of TRα1 and TRα2 mRNA and down-regulation of TRβ1 expression and this expression pattern is reversed during hyperthyroidism (7). In rats physiological hypertrophy shows only up-regulation of TRβ1 while during pathological hypertrophy all TR mRNAs were down-regulated (8).

In the present study we investigated the time course and relationship between systemic TH status and cardiac TR isoform expression during the development of congestive heart failure (CHF). We determined the protein levels of the TR isoforms and the mRNA level of the MHC isoforms and of SERCA2a in right and left ventricles of rat hearts in which RV pressure overload was induced by a single injection of monocrotaline (MCT). The pyrrolizidine alkaloid monocrotaline produces chronic pulmonary hypertension followed by the development of RV hypertrophy.

**Materials and Methods**

**Animals**

All animals were treated according to the national guidelines and with permission of the Animal Experimental Committee of the Vrije Universiteit Medical Center, Amsterdam, The Netherlands. Male Wistar rats, weighing 170-190g (Harlan, Zeist, The Netherlands) were randomly assigned to the three experimental groups; group 1: controls, Group 2: CHF/2 weeks, and group 3: CHF/4 weeks.

**Experimental protocol**

After one week acclimatization, animals received a single subcutaneous injection of MCT 80 mg/kg (congestive heart failure = CHF). The control group was injected with saline. Animals were housed individually (250 cm² /animal), on a 12h light/dark cycle and received food and water *ad libitum*. Animals were weighed four times in the first two weeks and daily from day 14 onwards. Two or four
weeks after injection animals were killed (five per group) with an overdose of halothane and the heart was rapidly excised. Blood was collected via the vena hepatica with heparin as anticoagulant. If necessary, first a trabecula was isolated, then right ventricle (RV), left ventricle (LV) and septum were separated. All specimens were weighed and tissue was immediately snap frozen in liquid nitrogen. Material was stored at -80°C until further use.

**Analysis of protein expression**

Anti-TR isoform polyclonal and monoclonal antibodies were previously described [(16) and chapter 3]. As secondary antibody, either goat antirabbit IgG conjugated with horseradish peroxidase or goat antimouse IgM conjugated with horseradish peroxidase (Roche Molecular Biochemicals, Mannheim, Germany) was used for Western blots.

To perform the Western blots, whole cell extracts from ventricles were prepared by homogenizing of 100 mg tissue in 1 ml sucrose (0.25 M) solution containing Complete protease inhibitor (Roche Molecular Biochemicals) using a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) for 10 seconds at maximum speed. The protein concentration of these whole cell extracts was determined using the Bio-Rad Bradford protein assay reagent (Bio-Rad Laboratories Inc., Munich, Germany). Subsequently, 40 μg protein was loaded on a 10% SDS-PAGE gel. After blotting onto PVDF membrane (Milipore), the blots were blocked for 45 minutes in PBS containing 1% (wt/vol) casein hydrolysate (Roche Molecular Biochemicals) and 0.05% (wt/vol) Tween-20 (blocking buffer; at pH=8 for TRβ1 and TRα2 and at pH=5.2 for TRα2). Next, the blots were incubated for 1 hour at 22°C followed by overnight at 4°C with the primary antisera anti-TR isoform (1:250 dilution for TRβ1 and 1:50 dilution for monoclonal anti-TR). The blots were washed twice 5 minutes with PBS containing 0.05% (wt/vol) Tween-20. After 30–45 minutes of incubation with the secondary antibody (1:20,000 dilution) in blocking buffer, blots were washed 3 times 5 minutes and LumiLight substrate (Roche Molecular Biochemicals) was added. The signals were visualized and quantified using a Lumi-Imager (Roche Molecular Biochemicals). The signal intensities are expressed as the logarithm of the light units per 40 μg whole cell extract.

**RNA-isolation and Slot-bLOTS**

Total RNA was isolated according to the method of Chomczynski and Sacchi (17). Briefly, frozen tissue was homogenized in RNAzol (Campro Scientific). RNA was isolated and quantified and the integrity was verified by gel-electrophoresis. Serial dilutions of RNA were applied to nylon membranes (Hybond N+, Amersham) using a Vacuum Slot-Blot system (BioRad). Duplicate blots, containing RNA of all experimental groups, were then hybridized with cDNA probes for SERCA2a (18), MHCα and -β (19) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Hybridization with cDNA probes was performed overnight at 42°C in buffer containing 50% formamide, 5 x SSC, 5 x Denhardt’s solution, 0.5% SDS and 50 mg/ml denatured salmon sperm DNA. The cDNA probes were labeled using the High prime labeling kit (Roche). A 32P-labeled dCTP was included in the reaction.
to obtain a specific activity of $1-5 \times 10^5$ /mg DNA. Blots were washed in 2 x SSC/0.1% SDS and 0.1 x SSC/0.1% SDS at 42°C. Membranes were exposed to Phosphor imager screens, scanned, and analyzed using the ImageQuant software (Molecular Dynamics). Quantification was performed by integrated optical density increase over background density in the rectangular region of interest. Data were expressed as the densitometric intensity of signals in arbitrary units relative to the GAPDH signal.

**Analysis of hormone data**
Plasma concentration of triiodothyronine ($T_3$) was determined with an in-house RIA (20).

**Statistical analysis**
hormone data were analyzed by unpaired two-tailed t-test. Due to its nonuniform distribution TR protein values were normalized by logarithmic protein concentrations followed by analyzed by unpaired two-tailed t-test. A difference was considered significant at $p < 0.05$. All data were evaluated using SPSS version 11 (SPSS, Inc., Chicago, IL).
Results

Development of heart failure
All animals treated with MCT eventually showed clear signs of CHF (i.e. low hormone values and decrease in the body weights). After two weeks no signs of heart failure were detectable in the treated group, while after three weeks some animals group developed the first signs of heart failure such as loosing weight between days 14-18 (data not shown). As a result, CHF animals had significantly lower body weights compared to controls (Table I). The lung weight was also significantly increased in the MCT-treated group compared to controls (Table I). This increase in lung weight is the result of proliferative pulmonary vasculitis rather than edema formation considering that dry/wet weight ratios of the lungs were not different between the experimental groups at any time point (data not shown). There was also a significant reduction in liver weight in CHF-rats compared to controls after four weeks (Table I). The liver weight was not different after two weeks, suggesting that the decreased liver weight was secondary to the development of heart failure. The ratio of RV over left ventricle plus septum (LV+S) was significantly higher in MCT-treated groups compared to controls already after two weeks, indicating the development of RV hypertrophy (Table I).

Table I. Changes in body and organ weights

<table>
<thead>
<tr>
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<th>control</th>
<th>MCT</th>
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<tbody>
<tr>
<td></td>
<td>BW (g)</td>
<td>liver (g)</td>
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<tr>
<td>2 weeks</td>
<td>282±18</td>
<td>11.7±0.6</td>
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<tr>
<td>4 weeks</td>
<td>327±23</td>
<td>14.4±1.2</td>
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BW: body weight, RV: Right ventricle, LV: left ventricle, S: septum. MCT: monocrotaline. Data are expressed in means ± SD. *P<0.010, **P<0.0010.
Thyroid hormone status
We determined the plasma levels of T₃ and T₄ at two and four weeks after MCT treatment. Both T₄ and T₃ decreased significantly in a time-dependent manner in the CHF group compared to controls, resulting in a 60% decrease (p<0.001) in both plasma T₃ and T₄ four weeks after MCT treatment (figure 1), which indicates the development of non-thyroidal illness (NTI).

![Figure 1](image)

**Figure 1.** Changes in the plasma levels of T₃ (left panel) and T₄ (right panel) at two and four weeks after MCT treatment. Data are expressed in means ± SD. *p < 0.001

TR isoform protein expression in CHF
We determined the expression levels of the TR isoform protein using the polyclonal and monoclonal antibodies we developed recently (16). We observed what appears to be a ventricle-specific effect on TR isoform expression as a result of CHF. No effect of MCT treatment was seen on TRβ1 protein levels in the LV, both at 2 weeks and 4 weeks. In contrast, a decrease in TRβ1 protein expression was already observed in the RV at 2 weeks which became more pronounced at 4 weeks (upper panel in figure 2). The effect on the TRα isoforms was the opposite with a gradual decrease of the protein expression of both TRα isoforms in LV and no significant effect in RV (lower panel in figure 2).
Target gene expression
To investigate the relationship between TRβ1 expression and the expression of SERCA2a and the MHCβ isoforms we determined the mRNA levels of these genes in RV and LV tissue two and four weeks after MCT administration. In LV, the SERCA2a mRNA was unchanged after two weeks but significantly decreased by 30% after four weeks. In RV, SERCA2a mRNA remained unaltered after two weeks, but decreased significantly after four weeks by 63% in the CHF group (lower panel figure 3). There was a shift in the expression of the MHC isoforms from the MHCα to the MHCβ isoform. The ratio of MHCα to MHCβ mRNA was significantly decreased in LV and RV at two weeks (0.4 in LV and 0.3 in RV).
whereas after four weeks MHC_\alpha mRNA was almost completely absent in CHF rats, while MHC_\alpha/MHC_\beta ratios were as low as 0.06 for LV and 0.02 for RV.

**Figure 3.** The ratio of MHC_\alpha to MHC_\beta mRNA (A) and SERCA2A mRNA expression (B) in right ventricle (RV) and in left ventricle (LV) in controls and at two and four weeks after monocrotaline treatment. Data are means ± SEM. The number of animals in each experiment are controls n = 6, n = 7 at two weeks and n = 4 at four weeks. * p < 0.05 and *** p < 0.001 versus controls.

**Discussion**

In this study we show the changes in TR isoform protein expression during the development of congestive heart failure. There appears to be a ventricle-specific effect on the expression of the TR isoforms with CHF affecting the TR_\alpha isoforms in LV and the TR_\beta1 in the RV. Similar results have been reported for the TR isoform mRNAs in a recent study of isoform mRNA expression in the left ventricle of the diseased human heart (7). However, an increase in the levels of the TR_\alpha2 isoform protein could not be demonstrated in this study whereas in the human heart an increase in the mRNA level of this isoform has been reported (7). It has been suggested that diminished TH signaling due to changes in TR expression could account for the gene expression pattern seen in heart failure, even under euthyroid conditions (9). In a rat model of cardiac hypertrophy, which did not show changes in plasma TH levels, all TR isoforms were indeed decreased...
in their expression (8). We would agree with these results in so far that during the TR isoform expression and concomitantly the MHC expression appears to decrease at least in the RV before any changes in plasma TH levels are detected. Further changes in both LV and RV are however also dependent on the decreased availability of TH as a result of the NTI.

In both ventricles changes were found in the expression of SERCA2a and in the MHC isoforms. It would therefore appear from our data that there is no strict relationship between MHCβ and the TRβ1 isoform as has been suggested elsewhere (8). The fact that we did not find this strict relationship is supported by the fact that studies in knock-out animals have shown that both SERCA2a and MHC are dependent both on TRα and TRβ albeit that there appears to be a preference of MHCβ for TRα1 (21,22). Parallel to the changes in TR isoform protein expression, a decrease in thyroid hormone levels is seen in the CHF animals indicating the development of NTI. Therefore, the effects on SERCA2a and MHC-isoform mRNA levels in late stage CHF will probably be the result of these reduced thyroid hormone levels in serum as well as the decrease in TR isoform protein levels. It is interesting to note that although the LV in our experimental system does not show signs of hypertrophy, gene expression is affected. Similar effects in LV in the absence of a direct pathological stimulus have been seen in this model in regard to alterations in the β-adrenoreceptor-adenylate cyclase system (23-25). There, the authors speculate that the changes in the LV are due to a neuro-endocrine overstimulation due to the right-sided heart failure.

In conclusion, we have demonstrated a ventricle specific change in TR isoform expression as a result of the development right ventricular hypertrophy that can eventually lead to heart failure. These changes in isoform expression are reflected in the expression of TH target genes and indicates that there appears to be, at least in our model, no clear connection between a particular target gene and a particular TR isoform.

Acknowledgement
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