Expression of thyroid hormone receptor isoforms in rodents

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CHAPTER 6

Hyper- and Hypothyroidism Change the Expression and Diurnal Variation of Thyroid Hormone Receptor Isoforms in Rat Liver Without Major Changes in their Zonal Distribution

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Submitted for publication
Abstract

We investigated the effect of hypothyroidism or hyperthyroidism on mRNA and protein expression of thyroid hormone receptor (TR) isoforms TRα1, TRα2 and TRβ1 in rat liver. Additionally we studied whether diurnal variation and zonal distribution of these isoforms are affected by changes in thyroid hormone supply. To this end hypothyroid, euthyroid and hyperthyroid rats were sacrificed at 13:30 and 19:30 h, time points chosen since they showed the largest difference in protein expression (chapters 2 and 3).

We found that hypothyroidism results in general in increased isoform mRNA and protein expression of TRα1, TRα2 and TRβ1, whereas in hyperthyroidism a decreased TRα1 and TRα2 mRNA and protein expression is seen. Interestingly during hyperthyroidism no change in TRβ1 mRNA is observed, but the TRβ1 protein is up-regulated in the light period and down-regulated in the dark period. Diurnal changes in the TR isoform proteins are abolished in hypothyroidism and hyperthyroidism, with the exception of a reversal in diurnal changes of TRβ1 in hyperthyroidism. Zonal distribution of the isoforms is not or minimally affected by hypo- or hyperthyroidism.

In conclusion this study demonstrates that the changes in expression of the different TR isoform mRNAs and proteins as a result of thyroid hormone depletion or excess tend to follow the same direction. Further we show that changes in the thyroid hormone state disregulate diurnal changes in TR protein expression.

Introduction

Thyroid hormones (TH; L-tetraiodothyronine; Thyroxine, T4; and L-triiodothyronine, T3) have a profound effect on liver function. For instance, thyroid hormones stimulate gluconeogenesis and glucose production in the liver and thus oppose the action of insulin with respect to hepatic glucose production (1). Hyperthyroidism worsens glycemic control in diabetic patients (2) and hypothyroidism results in decreasing glycogen breakdown, and gluconeogenesis (3). Most of the effects of thyroid hormones at the genomic level are mediated by binding of the hormone to thyroid hormone receptors (TR). After binding of thyroid hormone to its receptor, the receptor activates or represses the target gene. TRs are encoded by two different gene loci, TRα and TRβ. The TRβ locus encodes TRβ1, TRβ2, TRβ3 and also the truncated receptor TRΔβ3 (unable to bind thyroid hormone) which are all generated via alternative splicing or different promotor usage (4,5). The TRα gene encodes for TRα1 (able to bind thyroid hormone) with the splicing variant TRα2 and the truncated products TRΔα1 and TRΔα2 (6-8).

Previous studies indicate that TR isoform mRNA and protein expression are differentially regulated by thyroid hormones. During hyperthyroidism in rat livers, TRβ1 mRNA levels were unaffected while TRα1 and TRα2 were down-regulated (9). In the pituitary gland on the other hand, no change has been found
in TRα1, TRα2 and TRβ1 levels as a result of either hypo- or hyperthyroidism (10). In the heart no change was found in TRα1 and TRα2 mRNA levels as a result of hypo or hyperthyroidism and TRβ1 was up-regulated during hyperthyroidism (11). Furthermore the calculated TR isoform protein/mRNA showed a wide variation (10,12).

In a previous study we showed preferential expression of TRβ1 protein in the pericentral zone of rat liver, and a marked diurnal variation with the highest expression at the beginning of the dark period when rodents are mostly active (13). We also observed zonal expression of TRα1 and TRα2 in the liver of rat and mouse with the difference that the expression of TRα1 and TRα2 was broader than that of TRβ1 underlining the importance of local availability of TR isoforms. The zonal expression of TRs, combined with results obtained from TR isoform specific knock-out animals (14-17) suggests that TR isoform specificity in the regulation of for instance the CYP7A1 (only TRβ1 dependent) and deiodinase type 1 (mainly TRβ1 dependent) genes is thus dependent not only on the regulatory elements such as TRE in the target genes, but also on the factors governing the localised expression of the receptors isoforms.

Against the background of the zonal expression and the diurnal variation of the TR isoforms and their role as transcription factors in hepatic gene regulation we performed the present study to examine the pattern of TR expression at the mRNA and protein level in response to changes in thyroid state. We induced hyperthyroidism and hypothyroidism in rats and then analyzed mRNA expression using real-time quantitative PCR and quantified TR isoform protein expression by Western blotting and immunohistochemistry using our novel TR isoform specific antibodies.

**Materials and Methods**

**Experimental animals**

Male Wistar rats (Harlan, Horst, The Netherlands) were kept in 12/12, light/dark cycle (light on at 7:00 am) with free access to food. Rats were sacrificed (n=6/group) at 13:30h and 19:30h. Hypothyroidism was induced by a 21-day treatment with 0.05% propylthiouracil (Sigma, St. Louis, MO) in drinking water as described (18), and hyperthyroidism was induced by daily intraperitoneal injections of L-thyroxine (2mg/kg body weights; Roche Molecular Biochemicals, Mannheim, Germany) diluted in 5mM NaOH during three days (19). Liver and blood were collected for analysis. All studies were approved by the local animal welfare committee.

**Thyroid function tests**

Plasma Free T4 (FT4) and TSH were measured by immunoassays; FT4 was determined with a solid phase time–resolved fluoroimmunoassay (Delfia, Wallac Oy, Turku, Finland) and TSH plasma levels were determined using
chemiluminescent enzyme immunoassay (Immulite Third Generation TSH kit, rat TSH application, DPC, LA, California).

**Analysis of mRNA expression**

Poly-A RNA was isolated from each liver sample using a MagnaPure (Roche Molecular Biochemicals, Germany) according to the manufacturer's protocol. Thereafter, cDNA was synthesized using the First Strand cDNA synthesis kit with random primers (Roche Molecular Biochemicals). Real-time PCR reactions were performed in a LightCycler (Roche Molecular Biochemicals). TRα1 and TRα2 were simultaneously detected using sequence-specific hybridization probes and a LightCycler-FastStart DNA Master hybridization probes kit; probes and primers and program were previously described (20). A 185 bp specific fragment of TRβ1 was amplified using SybrGreen and the following primers sense: 5'-TGGGCGAGCTCTATATTCCA-3'; antisense: 5'-ACAGGTGATGCAGCGATAGT 3' (Gene Bank accession number gi:18855115).

As a housekeeping gene we used GAPDH of which a 430 bp fragment was amplified using a LightCycler-FastStart DNA Master Sybergreen kit and the primers sense 5'-AACCACGAGAAATATGACAAC-3'; antisense: 5'-CATCCTGGGCTACACTGAG-3' (Gene Bank accession number gi: 8393417). For each mRNA assayed a standard was generated and used in the range of 3pg to 0.3fg per 20 µl reaction mix. All results were normalized to the amount of GAPDH of each liver.

**Analysis of protein expression**

Anti-TRβ1 polyclonal antibodies were previously described (13). Anti-TRα1 and Anti-TRα2 monoclonal antibodies were raised against amino acids 402-410 (NH2-VFEDQEV-COOH) for TRα1 and amino acids 425-442 (NH2-LRGPVQLHQSPKSPQR-COOH) for TRα2.

Both monoclonal antibodies were isotyped as IgM. As secondary antibodies, goat antirabbit IgG+M and goat antimouse conjugated with alkaline phosphatase (DAKO Corp., Glostrup, Denmark) were used for immunochrometry, and goat antirabbit IgG and goat antimouse conjugated with horseradish peroxidase (Roche Molecular Biochemicals, Mannheim, Germany) were used for Western blots.

To perform the Western blots, whole cell extracts from livers were prepared by homogenisation of 500 mg liver in 5 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 a Bradford reagent obtained from Bio-Rad Laboratories, Inc. (Munich, Germany). Subsequently, 25 µg protein was loaded on a 10% SDS-PAGE gel. After blotting onto PVDF membrane (Milipore PM), 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 first for 1 hour at 22°C followed by overnight at 4°C with the primary antiserum anti-TRβ1 (1:250 dilution) or monoclonal anti-TRα1 or TRα2 (1:50 dilution). The blots were washed 2X 5 minutes with PBS containing 0.05% (wt/vol) Tween-20. After 30-45 minutes of incubation with secondary antibodies (1:20,000 dilution) in blocking buffer, the blots were washed 3X 5 minutes and LumiLight™ substrate (Roche Molecular Biochemicals) was added. The light unit signals were visualized and quantified using a Lumi-Imager (Roche Molecular Biochemicals).

Immunohistochemistry and image analysis
For immunohistochemistry, 6-μm thick, paraformaldehyde-fixed (4% in PBS for 16 hours at 4°C), paraffin-embedded liver sections were subjected to microwave irradiation (Tris-buffered saline (TBS), pH=7.6, at 750 watts for 10 minutes) before immunodetection to unmask the antigenic epitope. The sections were incubated with antiserum (1:250 dilution for TRβ1 and 1:20 or TRα2 in TBS (pH=7.6), 5% (wt/vol) low fat milk, and 0.5% (wt/vol) Triton X-100 and for TRα1 1:20 dilution in 0.25 mM acetate buffer (pH=5.2), 1% (wt/vol) low fat milk, and 0.5% (wt/vol) Triton X-100 for 1 hour at 22°C and then overnight at 4°C. Secondary antibody (1:250 dilution) incubation was performed for 60 minutes at 22°C in PBS (pH=7.6) containing 0.5% (wt/vol) Triton X-100. The sections were then washed and incubated in buffer (0.1 M Tris, 0.1 M NaCl, and 50 mM MgCl₂; pH=10.2) for 10 minutes. Twenty minutes after adding nitro blue tetrazolium 5-bromo-4-chloro-3-indolyl-phosphate (Roche Molecular Biochemicals) and levamisole (Sigma), the staining reactions were stopped by washing with TBS. Zonal expression of TRs in liver sections was scored by three masked independent observers on a pre-defined scale, from 0 to 2; 0 = Homogenous distribution, 1 = broad zonal expression and 2 = sharp zonal (pericentral) expression. Scores of the these observers were afterwards averaged and subjected to statistical analysis.

Statistical analysis
Differences between groups were analyzed by the Mann-Whitney-U non-parametric test. A difference was considered significant at P < 0.05. All data were evaluated using SPSS version 11 (SPSS, Inc., Chicago, IL).

Results
Hormone measurements
Plasma concentrations of FT₄ and TSH in animals confirmed the induction of either hyper or hypothyroidism (Table 1). Hypothyroid animals were also distinguished from the other groups by lower body weight as shown in table 1. In hypothyroid rats, FT4 and TSH levels differed compared to euthyroid animals with higher TSH levels and lower FT4 levels. In hyperthyroid animals the opposite effect, namely higher FT4 and lower TSH, was found. FT4 and TSH
levels did not differ between rats sacrificed at 13:30 h or 19:30 h, with the only exception of higher TSH level at 19:30 h in hypothyroid rats.

Table 1. Thyroid function tests and body weight of hypothyroid, euthyroid and hyperthyroid animals (n=6 in each group) during the Light (13:30, ZT6.30) and Dark (19:30, ZT12.30) period. (Values in means ± SD).

<table>
<thead>
<tr>
<th>Light period (13:30 h)</th>
<th>TSH (ng/ml)</th>
<th>FT4 (pmol/L)</th>
<th>Body weight (grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothyroid</td>
<td>26.9 ± 0.2*</td>
<td>2.9 ± 2*</td>
<td>312 ± 8*</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>2.3 ± 1.5</td>
<td>37.7 ± 6.7</td>
<td>390 ± 18</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>0.50 ± 0.1*</td>
<td>&gt;70†</td>
<td>369 ± 11†</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dark period (19:30 h)</th>
<th>TSH (ng/ml)</th>
<th>FT4 (pmol/L)</th>
<th>Body weight (grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothyroid</td>
<td>34.9 ± 5.3*</td>
<td>2.8 ± 0.4*</td>
<td>295 ± 8*</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>1.6 ± 1.1</td>
<td>43.6 ± 11.7</td>
<td>375 ± 21</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>0.5 ± 0.1†</td>
<td>&gt;70†</td>
<td>369 ± 16†</td>
</tr>
</tbody>
</table>

* p=0.004, † p=0.002 and , ‡ p=0.016 and * p=0.04 versus euthyroid animals.

Expression of TR isoform mRNA and protein and thyroid status
TRα1 mRNA expression was higher in hypothyroid and lower in hyperthyroid animals when compared to euthyroid animals both at 13:30 and 19:30 time points. TRα1 protein expression was higher in hypothyroid rats at 13:30 h but not at 19:30 h and lower in hyperthyroid animals at 19:30 h but not at 13:30 h. (figure 1, upper panel).

TRα2 mRNA expression was also higher in hypothyroid animals and lower in hyperthyroid animals as compared to euthyroid animals at 13:30 h; the same trend was observed at 19:30 h but failed to reach significance. TRα2 protein expression was likewise higher in hypothyroid and lower in hyperthyroid animals at both time points (figure 1, middle panel).

TRβ1 mRNA expression was higher in hypothyroid rats relative to euthyroid rats at both 13:30 and 19:30 h; it did not change in hyperthyroid rats. TRβ1 protein expression in the hypothyroid rats was higher only at 13:30 h, whereas in hyperthyroid animals it was higher at 13:30 h but lower at 19:30 h (figure 1, lower panel).
Figure 1. Effect of thyroidal state on the tissue mRNA and protein levels of TRα1, TRα2 and TRβ1 during the Light (13:30, ZT6:30) and Dark (19:30, ZT12:30) period in rat liver. The results are presented in "box and whisker" plots. The box represents the interquartile range which contains 50% of the values. The whiskers are lines that extend from the box to the highest and lowest values. Ho, hypothyroid; eu, euthyroid; hr, hyperthyroid animals. #, p<0.05; $, p<0.01; * p<0.001 versus euthyroid animals.
Expression of TR isoforms and their diurnal variation

No significant changes were found at the mRNA level for either of the three isoforms when comparing the light and dark time points under the different thyroid states.

Next we looked for diurnal changes in the protein levels of each receptor isoform (Table 2). Both TRα1 and TRα2 showed a diurnal variation at the protein level in euthyroid animals but with opposite direction; the TRα1 levels were highest in the dark period and lowest in the light period with the reverse seen for TRα2. Although hypo- and hyperthyroidism both eliminated the diurnal variation for TRα1 and TRα2, the protein levels differed between hypo- and hyperthyroid animals (see above and Table 2).

In euthyroidism, diurnal variation was most profound for TRβ1 protein with the highest level during the dark period (Table 2), as reported before (13). The daily variation in the levels of this isoform disappeared in the livers of hypothyroid rats and, interestingly, seems to switch during hyperthyroidism with levels during the light period being the highest.

Table 2. Diurnal variation of TR isoform proteins in hypothyroid, euthyroid and hyperthyroid animals (n=6 in each group). Values are arbitrary units expressed as median ± range.

<table>
<thead>
<tr>
<th></th>
<th>TRα1</th>
<th>TRα2</th>
<th>TRβ1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothyroid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13:30</td>
<td>85.7 (68.0-179)</td>
<td>112.7 (58.1-176.2)</td>
<td>104.3 (95.2-120.2)</td>
</tr>
<tr>
<td>19:30</td>
<td>87.1 (57.0-170.7)</td>
<td>81.6 (22.4-106.6)</td>
<td>148.5 (99.1-161.4)</td>
</tr>
<tr>
<td>Euthyroid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13:30</td>
<td>59.6 (54.6-75.9)</td>
<td>67.6 (51.1-76.8)</td>
<td>63.4 (54.7-73.8)</td>
</tr>
<tr>
<td>19:30</td>
<td>84.0 (77.9-120.3) *</td>
<td>41.0 (25.9-52.4) +</td>
<td>118.2 (105.6-154.2) †</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13:30</td>
<td>82.9 (13.2-116.6)</td>
<td>16.6 (14.0-50.4)</td>
<td>117.4 (103.7-135.3)</td>
</tr>
<tr>
<td>19:30</td>
<td>55.2 (38.0-87.6)</td>
<td>22.3 (9.3-42.2)</td>
<td>85.9 (52.5-104.3) +</td>
</tr>
</tbody>
</table>

† p=0.004, * p=0.006 and + p=0.01; 19:30 versus 13:30 h.

Zonal distribution of TR proteins

We reported earlier on the fact that TRβ1, TRα1 and TRα2 are zonally expressed around the central veins of the rat liver. In this study we performed immunohistochemistry to investigate whether the TR isoform localization and zonal expression is affected by the thyroid state. The micrographs in figure 2 show that thyroid hormone deficiency or excess are not associated with obvious changes. When scored by three masked observers, the zonal expression of TRβ1 and TRα1 remained unchanged, while TRα2 zonation was slightly broader in hyperthyroid animals in the light period (Table 3).
Figure 2. Localisation of TRβ1, TRα1 and TRα2 in sections of the livers of hypothyroid, euthyroid or hyperthyroid animals during Light (13:30, ZT6.30) period. C: central veins, P: portal veins. Bar, 100 μm. Hypo, hypothyroid; eu, euthyroid; hypr, hyperthyroid animals.
Table 3. Changes in zonal expression of TR isoforms in rat liver hypothyroid, euthyroid and hyperthyroid animals (n=6 in each group) during the Light (13:30, ZT6.30) and Dark (19:30, ZT22.30) period. (Values in means ± SEM).

<table>
<thead>
<tr>
<th>Light period (13:30 h)</th>
<th>TRα1</th>
<th>TRα2</th>
<th>TRβ1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothyroid</td>
<td>1,1 ± 0.2</td>
<td>1.4 ± 0.3</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>0.8 ± 0.3</td>
<td>1.8 ± 0.1</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>1.0 ± 0.2</td>
<td>1.3 ± 0.2*</td>
<td>2 ± 0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dark period (19:30 h)</th>
<th>TRα1</th>
<th>TRα2</th>
<th>TRβ1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothyroid</td>
<td>0.8 ± 0.2</td>
<td>1.4 ± 0.3</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>0.7 ± 0.4</td>
<td>1.4 ± 0.2</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>0.7 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>2 ± 0</td>
</tr>
</tbody>
</table>

Scores from 0 to 2 (0, homogeneous distribution; 1, broad zonal expression; 2 sharp zonal expression). *p=0.025 versus euthyroid animals.

Discussion

The effects of thyroid state on tissue levels of TRs were investigated by induction of hypothyroidism and hyperthyroidism. Studies looking at the effect of thyroid state on T3 nuclear-binding capacity, isoform mRNA and protein content of tissues and cell lines have yielded paradoxical results in the sense that (changes in) mRNA levels were found without concomitant (changes in) protein and vice versa (9,10,21,22). Since we have shown that the expression of TR isoform protein has a diurnal rhythm ((13), and this paper) whereas no clear diurnal variation is seen in the mRNA expression. One of the reasons for these discrepancies could be that the animals were killed at a time point when mRNA expression was high but protein levels were low. This phenomenon has been demonstrated for the per gene product in Drosophila were the protein levels peak 4-6 h after the mRNA peak (23).

The results obtained in this study indicate that hypothyroidism generally resulted in up-regulation of all TR isoform mRNA levels at both time points i.e., light and dark periods. These results agree with those reported earlier (9,22). The increase in the mRNA levels is accompanied by an increase in TR isoform protein levels only during the light period (13:30 h), this can be explained by the fact that according to our data the diurnal rhythm during hypothyroidism seems to disappear with always high TR levels.

Hyperthyroidism results in down-regulation of TRα1 and TRα2 mRNA during the light period when compared to the euthyroid situation and, although no significant changes were found in the dark, there appears to be a trend towards a decrease at this time point as well. Hyperthyroidism did not influence TRβ1 mRNA expression in liver, which is similar to the results reported earlier (9). Again, as in the case of hypothyroidism, hyperthyroidism resulted in a loss of the diurnal rhythm for TRα1 and TRα2 with overall levels of TRα2 being lower.
than in the case of euthyroidism. An interesting phenomenon seems to occur in
the case of TRβ1 where diurnal variation is not lost during thyroid hormone
excess but instead, the rhythm appears to have reversed with the highest value
during the light period instead of the dark period.
In general, protein levels tend to follow the mRNA expression in our
experiments apart from the TRβ1 protein the levels of which rise during both
hyper- and hypothyroidism, which in the former situation is not accompanied by
an increase in the mRNA. The time points we used were chosen for their
highest difference in protein levels (13) so this discrepancy could be explained
by the aforementioned fact that in diurnal processes an mRNA rise is found a
few hours before the protein rise. Another possibility could be a change in the
stability of the protein which can take place as a result of changes in phosphorylation
(24) or differences in the proteasome degradation pathway of the TRβ1 (25).
Immunohistochemistry showed that changes in the thyroid state in general had
no influence on the localization of TR isoforms which remained restricted to the
same zones of rat livers, although hyperthyroidism appeared to result in a
somewhat broader zonation of TRα2 at 13:30h. These results indicate in
general that any changes in the expression of T3-dependent genes, for instance
an increase, will be the result of an increased expression in the same cells in the
same zone, not an involvement of more cells outside the zone.
We have recently shown that the zonal expression of TRβ1 is overlaps with the
zonal expression of "Spot 14" which has been shown to be TRβ1 isoform-
dependent. One could also expect that the diurnal rhythm in TR isoform
expression is reflected in the expression of certain T3-dependent genes.
Tentative examples could be the diurnal rhythm of the thyroid hormone
responsive and TRβ1-dependent Cyp7a and "Spot 14" genes or the thyroid
hormone-dependent HMGCoA-reductase gene. Both have high levels during
the dark period matching the TRβ1 rhythm.
In conclusion we show in this study that the changes in expression of the
different TR isoform mRNAs and proteins as a result of thyroid hormone
depletion or excess tend to follow the same pattern. Further, we show that
changes in the thyroid hormone state deregulate diurnal changes of TR protein
expression. Further studies are aimed at clarifying the biologic relevance and
nature of the changes in the diurnal variation of TR in respect to their effect on
hepatic gene regulation under different hormonal conditions.

Acknowledgement
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Organisation for Scientific Research (NWO)
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