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Triiodothyronine affects the alternative splicing of thyroid hormone receptor alpha mRNA

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Abstract

The c-erbAα gene encodes two thyroid hormone receptors, TRα1 and TRα2 that arise from alternative splicing of the TRα pre-mRNA. TRα2 is not able to bind T₃ and acts as a weak antagonist of TRs. It has been suggested that the balance of TRα1 to TRα2 is important in maintaining homeostasis. Here we study the effect of thyroid hormone on the splicing of TRα under various conditions in HepG2 cells. First T₃ was added to HepG2 cells that endogenously express TRα. This resulted in a decrease in the TRα1:TRα2 mRNA ratio by the addition of 10⁻⁹M or 10⁻⁷M T₃. Then HepG2 cells were incubated with sera from hypothyroid or hyperthyroid patients. Sera from hyperthyroid patients (n=6) decreased the TRα1:TRα2 ratio compared to HepG2 cells incubated with sera from euthyroid patients (n=8). Sera from hypothyroid patients (n=6) had no effect on the TRα1:TRα2 ratio but supplementation with T₃ caused a decrease in the ratio. Finally we tested sera from patients with nonthyroidal illness (n=17) which showed no effect on TRα splicing when compared to controls. fT₄ levels of sera from hypo-, eu-, and hyperthyroid patients, but not of NTI patients, were negatively correlated (p<0.01) to the TRα1:TRα2 ratio. We next studied the expression of splicing factors hnRNP A1 and ASF/SF2 (SF2) in relation to the splicing of the TRα gene. In HepG2 cells incubated with NTI sera a negative relationship was found between the ratio of hnRNP A1:SF2 and the TRα1:TRα2 ratio. A high hnRNP A1:SF2 ratio is associated with the use of the distal 5'splice site. The splicing direction should then change towards TRα2, which is indeed the case. Rev-ErbA which is partly complementary to TRα2 and could therefore interfere in the splicing process did not relate to the TRα1:TRα2 ratio.

In conclusion, high T₃ levels induce a low TRα1:TRα2 ratio which could protect the cell from excessive T₃ induced gene expression. In vivo this might be a mechanism to keep tissues relatively euthyroid during high serum T₃ levels.

Introduction

Thyroid hormone exerts its actions primarily by binding to thyroid hormone receptors (TRs) that bind to thyroid hormone response elements in the regulatory region of a gene thereby modifying gene expression. Five different TR isoforms are known (α1, α2, β1, β2, β3) which are derived from the genes c-erbAα and c-erbAβ respectively (1;2). The c-erbAα gene encodes two variants that differ at their C-terminal end by the
use of an alternative splice site in exon 9. TRα1 is a functional receptor that binds T₃ whereas TRα2 does not bind T₃ and may have a dominant negative effect. TRα2 can exert its dominant negative activity either by competing for TR binding to thyroid hormone responsive elements (TREs) on DNA (3;4) or via other mechanisms that do not require binding to a TRE(5).

TRα₂⁻/⁻ mice overexpress TRα1 and are hypothyroid but show a complex phenotype with features of both hypo- and hyperthyroidism (6). A disturbance in the balance of the TRα₁:TRα₂ ratio may be responsible for the changes in thyroid hormone sensitivity of the different tissues. Moreover, mice that lack all TRα isoforms have an increased sensitivity to thyroid hormone, possibly due to the absence of the silencing effect of the dominant negative TRα2 (7). We hypothesized that T₃ might have an effect on the alternative splicing of TRα mRNA by changing the balance towards TRα2 in case of high T₃ levels. This might then counterbalance excessive T₃ induced gene transcription during hyperthyroid conditions. We therefore tested the effect of T₃ addition on endogenously expressed TRα1 and TRα2 in HepG2 cells. Subsequently we also looked at the effect of adding sera from patients who were hyperthyroid or hypothyroid. We use HepG2 cells as a model, for these cells have previously been used to study the uptake, metabolism and action of thyroid hormone and have proven to be a suitable model for thyroid hormone handling by the human liver (8;9). Induction of the low T₃ syndrome in rats by fasting alters the balance between TRα₁ and TRα₂ in liver due to an increased expression of TRα₂ (10). This leads to the hypothesis that serum from patients with nonthyroidal illness (NTI) might also have an effect on the splicing process of TRα and therefore we also tested sera of NTI patients in our HepG2 model.

It is likely that there is a control of TRα₁ and TRα₂ mRNA levels by regulation of alternative splicing of the TRα pre-mRNA and splicing factors may play a role in the selection of the alternative splice site of the TRα pre-mRNA. Certain components of the general splicing mechanism, such as the family of serine-arginine (SR) proteins affect splice site selection in a dose dependent manner; they are counteracted by hnRNP A1 and related proteins (11;12). The ratio of SR proteins and hnRNP A1-like proteins is therefore an important determinant for alternative 5' splice-site selection in transfected cells (13). The identification of a splicing enhancer element (SEα₂) within the final intron of TRα₂ mRNA that stimulates TRα₂ mRNA splicing and interacts with ASF: SF2 (SF2) supports the idea that these splicing factors are involved (14).
Another interesting feature of the erbAα locus is the presence of a third gene, Rev-ErbAα (RevErb), encoded on the opposite strand of the erbAα (15). The 3’end of RevErb overlaps with sequences coding for TRα2 but not TRα1. The fact that RevErb mRNA is partially complementary to TRα2 mRNA could indicate that it is involved in a possible regulatory mechanism for TRα mRNA processing. In vitro splicing towards TRα2 pre-mRNA is inhibited by addition of an excess of antisense RNAs containing the 3’end of RevErb mRNA (16). RevErb is also associated with an increase in the ratio TRα1 to TRα2 mRNA in differentiating adipocytes (17). Another study aim was consequently to evaluate possible involvement of SF2, hnRNP A1 and RevErb in the splicing process of TRα pre-mRNA.

Patients & Methods

Materials

T₃ was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in 5mM NaOH to a concentration of 1 mg/ml. It was further diluted in E/MEM (Biowhittaker, Verviers, Belgium) and stored at -20°C.

Cell culture

The human hepatoma cell line HepG2 was obtained from the ATCC (#HB 8065, American Type Culture Collection, Rockville, Maryland, USA). For the first experiment cells were cultured in EMEM supplemented with 10 U/ml penicillin/streptomycin/fungizone (p/s/f) and 5% fetal calf serum (all from Biowhittaker, Verviers, Belgium). Cells were plated in 6-wells plates and reached approximately 70% confluence after 18 hours. Medium was then changed to incubation medium with 5% FCS and an increasing amount of T₃ (10⁻⁹ to 10⁻⁷M). HepG2 cells were incubated with each concentration of T₃ in six-fold for a further 24 hours after which RNA was isolated.

The second experiment was performed with sera from hypothyroid, hyperthyroid and euthyroid controls. HepG2 cells were incubated in 24-wells plates containing E/MEM supplemented with 10 U/ml (P/S/F) and 10% serum. Each serum sample was tested in duplicate for 24 hours.

In the third experiment sera from two hypothyroid patients were tested in triplicate with increasing amounts of T₃ (10⁻⁹ to 10⁻⁷M).
In the fourth experiment we incubated HepG2 cells with E/MEM containing 10 U/ml (P/S/F) and 10% serum from NTI patients or healthy controls. We isolated cells after 5, 10 and 24 hours of incubation. All samples in this experiment were tested in triplicate. Sera were assayed in four separate runs; each run contained 2 serum samples from group I-III.

Patients

Sera were collected from 8 healthy volunteers, 6 hypothyroid (TSH>4 mU/l, fT4<10 pmol/l) patients and 6 hyperthyroid (TSH<0.4 mU/l, fT4>23 pmol/l) patients.

Additionally we collected sera from 24 patients hospitalized at the Department of Internal Medicine of our institute. Excluded were patients with thyroid or pituitary/hypothalamic diseases, or using drugs known to interfere with thyroid hormone metabolism or regulation. Diagnoses of the patients admitted to the study were infectious disease (n=7), renal insufficiency (n=6), cancer (n=5), liver disease (n=2), multiple sclerosis (n=1), gastrointestinal disease (n=2) and venous thrombosis (n=1). Sera from 8 healthy volunteers acted as controls in this experiment (group I). Sera of patients were divided in two groups: group II with normal serum T3 and T4 (T3≥1.3 nmol/l, T4≥75 nmol/l, n=7) and group III with subnormal T3 and normal or subnormal T4 levels (T3<1.3 nmol/l, n=17). Sera were stored at -20°C.

Assays

Serum T3 and T4 were measured with in-house RIAs (18). Free T4 was measured by a two-step fluoroimmunoassay (DELFIA; Wallac, Turku, Finland). TSH was measured with an immunofluorometric assay (DELFIA). A commercial enzyme immunoassay (Pelikine Compact human IL-6 ELISA kit, CLB, The Netherlands) was used to measure IL-6 with a sensitivity of 0.5-1 pg/ml.

RT-PCR

Total RNA was isolated with the RNeasy isolation system (Qiagen GmbH, Hilden, Germany) and reverse transcribed into single-stranded cDNA using the First Strand cDNA synthesis kit with random primers (Roche Molecular Biochemicals, Mannheim, Germany). Real-time PCR reactions were performed in a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany). TRa1 and TRa2 were simultaneously detected in the same sample using sequence-specific hybridization probes and a LightCycler-Fast-
Start DNA Master Hybridization Probes kit. Probes, primers and program were as previously described (19). β-Actin, hnRNP A1 and SF2 were measured using the LightCycler-DNA Master SYBR Green kit and RevErb with the LightCycler Faststart DNA master SYBR Green kit. We designed primer pairs for hnRNP A1 (sense: 5’-CATGACTGACCGAGGCAGTG-3’; antisense: 5’-GGCTGGATGAAGCACTAGCC-3’ and RevErb (sense: 5’-GCTCGGGAATGCAGAATTC-3’; antisense: 5’-CCAGAGGCTCATCTTGGAAT-3’). Specificity of the product was checked by gel electrophoresis. Primers for ASF: SF2 and β-actin were as described (20;21). For each mRNA assayed, a sequence-specific standard was generated and used in the range of 0.1-1000 fg/20 μl. The PCR reactions were cycled with the following programs: β-actin: denaturation at 95 °C for 10 s, 45 cycles of 0 s 95 °C, 5 s 52 °C, 10 s 72 °C, hnRNP A1: denaturation at 95 °C for 10 s, 45 cycles of 0 s 95 °C, 5 s 50 °C, 10 s 72 °C and SF2: 95 °C for 10 s, 45 cycles of 0 s 95 °C, 5 s 50 °C, 10 s 72 °C, 5 s 80 °C, all with a final MgCl₂ concentration of 5mM. The following program was used for measuring RevErb mRNA: 95 °C for 600 s, 45 cycles of 0 s 95 °C, 5 s 50 °C, 10 s 72 °C, 5 s 81 °C. Final concentration of MgCl₂ was 4mM. Melting curves were analyzed with continuous fluorescence reading. From the standard curve generated by measurements taken during the exponential phase of the amplification, the amount of mRNA in each sample was determined. All results were normalized to the amount of β-actin mRNA and are expressed as relative units.

Western Blots

HepG2 cells were cultured in 75 cm² flasks for 24 hours in E/MEM containing 5% FCS in the presence of 10⁻⁷ M T₃ or without T₃ (only diluted NaOH in E/MEM as a control). Cells were washed two times with ice-cold PBS and a whole cell extract was made by scraping the cells in 1 ml homogenization buffer (0.25M sucrose, 10 mM Hepes, 25mM KCl, 1mM EDTA, 10% glycerol containing spermine, spermidine and Complete protease inhibitor (Roche Molecular Biochemicals)). The cells were then disrupted at 6500 rpm for 60 s in a MagNA Lyser (Roche) with Green Beads (ceramic beads, Roche). Subsequently, 20 μl of protein suspension in loading buffer was loaded onto a 10% SDS-Page gel. TRα1 and TRα2 were detected as described by Zandieh Doulabi et al (in press) using monoclonal antibodies.

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Data analysis

Differences between groups were analyzed with the Mann-Whitney-U test. Pearson's coefficient of correlation was used for evaluation of the relationship between the TRα1:TRα2 ratio and the hnRNP A1: SF2 ratio and for correlations of serum T3, T4 and fT4 with the TRα1:TRα2 ratio. Spearman's correlation coefficient was used to analyze the correlation between IL-6 and T3.

Results

In the first experiment we tested if T3 addition had an effect on the endogenous TRα mRNA expression and/or the alternative splicing process of TRα in HepG2 cells. The amount of T3 in the FCS is in the picomolar range which is well below the Kd of the thyroid hormone receptor and therefore unlikely to cause interference. Incubation of cells with concentration of 10^-8 M and 10^-7 M T3 significantly decreased the ratio of TRα1 to TRα2 (Fig.1). The change in the TRα1:TRα2 ratio was confirmed on a Western Blot. After incubation of HepG2 cells with 10^-7 M T3 the TRα1:TRα2 ratio on protein level decreased from 0.98 to 0.61 (Fig 2).

The mRNA ratio of the splicing factors hnRNP A1:SF2 did not differ when T3 was added. There was also no effect of T3 on RevErb mRNA expression.

The second experiment was performed to evaluate the effects of human euthyroid, hyperthyroid or hypothyroid serum samples on the expression of TRα in HepG2 cells. Hyperthyroid sera contained lower levels of T3 and T4 compared to euthyroid sera whereas hyperthyroid sera had increased levels of T3 and T4 (Table 1). Incubation of HepG2 cells with hyperthyroid sera induced a decrease in the TRα1:TRα2 ratio whereas incubation with hypothyroid sera did not have an effect (Fig.3). No difference was found in total TRα mRNA expression between the three groups. We then analyzed the mRNA
expression of RevErEr and the splicing factors hnRNP A1 and SF2 and found no differences in expression between the three groups. Furthermore, there was also no correlation with the TRα1:TRα2 ratio. The 10-fold increase in the TRα1:TRα2 ratio when compared to the first experiment is caused by the addition of 10% human serum.

The third experiment was performed to test if the high concentration of T3 in serum of hyperthyroid patients caused the decrease in TRα1:TRα2 mRNA ratio in HepG2 cells. We therefore added increasing concentrations of T3 to hypothyroid human serum. After a 24 hour incubation of HepG2 cells with this T3-enriched serum, the TRα1:TRα2 ratio decreased significantly (Fig. 4).

With the fourth experiment we investigated whether the well-known altered concentration of T3 and T4 in serum of patients with NTI would have an effect on the splicing process of the TRα pre-mRNA in HepG2 cells. Thyroid function tests and IL-6 serum levels of NTI patients and controls are presented in Table 2. Serum IL-6 levels were elevated in the NTI patients when compared to healthy controls, but no differences could be detected within the NTI patient groups. When all patients were analyzed together, a

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**Table 1.** Thyroid function test of euthyroid, hypothyroid and hyperthyroid subjects. Values are given as median and range.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hypothyroid</th>
<th>Euthyroid</th>
<th>Hyperthyroid</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (M,F)</td>
<td>6 (0.6)</td>
<td>8 (3.5)</td>
<td>6 (0.6)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>35 (27-51)</td>
<td>38 (24-51)</td>
<td>45 (32-56)</td>
</tr>
<tr>
<td>Total T3 (nmol/l)</td>
<td>1.2 (0.5-2.1)</td>
<td>2.2 (1.8-3.0)</td>
<td>5.9 (2.7-7.9)</td>
</tr>
<tr>
<td>Total T4 (nmol/l)</td>
<td>35 (5-115)</td>
<td>110 (70-145)</td>
<td>245 (210-397)</td>
</tr>
<tr>
<td>Free T4 (pmol/l)</td>
<td>3.2 (2.0-9.6)</td>
<td>14 (12-20)</td>
<td>66 (26-70)</td>
</tr>
<tr>
<td>TSH (mU/l)</td>
<td>95 (12-226)</td>
<td>1.45 (0.5-2.3)</td>
<td>0.035 (0.02-0.05)</td>
</tr>
</tbody>
</table>

*aP<0.01, *bP<0.05, *cP<0.001: hypothyroid compared with euthyroid; *dP<0.001, *eP<0.05; hyperthyroid compared with euthyroid (Mann-Whitney U test).
T affects the splicing of TRα mRNA

Figure 3: The TRα1:TRα2 mRNA ratio in HepG2 cells incubated with sera from hypothyroid, euthyroid and hyperthyroid subjects. Values as mean ± SEM (n=6). P-values are obtained with the Mann-Whitney-U test.

Figure 4: The TRα1:TRα2 mRNA ratio in HepG2 cells incubated with sera from hypothyroid subjects, supplemented with increasing amounts of T3 (10^-9 M to 10^-7 M). Values as mean ± SEM (n=6); p-values are obtained with the Mann-Whitney-U test.

strong correlation was found between total serum T3 and serum IL-6 (Spearman's correlation coefficient, r=-0.61, p<0.001). We harvested HepG2 cells after 5, 10 and 24 hours of incubation with serum. Since no significant effects on the splicing of TRα were seen after 5 and 10 hours (data not shown) we only describe the data after 24 hours of incubation. The TRα1:TRα2 ratio did not change between cells incubated with sera from different groups (Fig. 5A). No differences were found between the different groups for any of the measured mRNAs with the exception of increased RevErb mRNA in NTI

Table 2. Thyroid function tests and serum IL-6 in controls (group I) and hospitalized patients with normal serum T3 (group II) or subnormal T3 (group III). Values are given as median and range.

<table>
<thead>
<tr>
<th>parameter</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (M,F)</td>
<td>8 (3,5)</td>
<td>7 (4,3)</td>
<td>17 (8,9)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>40 (24-59)</td>
<td>43 (18-58)</td>
<td>53 (27-79)</td>
</tr>
<tr>
<td>Total T3 (nmol/l)</td>
<td>2.2 (1.8-3.0)</td>
<td>1.6 (1.3-2.4)</td>
<td>0.6 (0.2-1.2) a,b</td>
</tr>
<tr>
<td>Total T4 (nmol/l)</td>
<td>98 (70-145)</td>
<td>110 (80-140)</td>
<td>75 (35-125) c</td>
</tr>
<tr>
<td>Free T4 (pmol/l)</td>
<td>14 (12-20)</td>
<td>15 (12-23)</td>
<td>16 (9-25)</td>
</tr>
<tr>
<td>TSH (mU/l)</td>
<td>1.5 (0.5-2.3)</td>
<td>1.5 (0.3-4.0)</td>
<td>2.2 (0.2-5.6)</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>1.1 (0.1-2.1)</td>
<td>16.3 (2.5-104.0) a</td>
<td>23.6 (3.9-654.0) a</td>
</tr>
</tbody>
</table>

aP≤0.001 vs group I, bP≤0.05 vs group II, cP≤0.001 vs group II (Mann-Whitney U test).
patients with a subnormal $T_3$ (Fig. 5B). The mRNA expression of RevErb was negatively correlated to the level of serum $T_3$ ($r=-0.39$, $p<0.05$). We did not find a relationship between the TRα1:TRα2 ratio and RevErb. Interestingly, although no effect of the individual splicing factors hnRNP A1 and SF2 on the TRα1:TRα2 ratio was seen, a negative correlation ($r=-0.40$, $p<0.05$) was found between the TRα1:TRα2 ratio and the ratio of hnRNP A1/SF2 (Fig. 6). No relation was found between IL-6 and any of the measured mRNAs.

Whereas a clear negative correlation was found between serum $fT4$ levels of hypothyroid, euthyroid and hyperthyroid patients and the effect of these sera on the TRα1:TRα2 ratio, this relationship was not observed for NTI sera (Fig 7).

**Figure 5:** HepG2 cells were incubated with sera from healthy subjects (group I) or NTI patients (group II and III) for 24 hours. Effect on the TRα1:TRα2 mRNA ratio (panel A) and on RevErb mRNA expression (panel B). Values as mean + SEM; $p$-values are calculated with the Mann-Whitney-U test.
Discussion

The balance between TRα1 and TRα2 is important since they are both transcription factors but have opposite effects on T₃-responsive gene expression (22). Studies in mouse strains that lack both TRα isoforms or only TRα2, have shown an increased sensitivity to T₃ which is mainly attributed to the abrogation of the constitutive silencing mediated through TRα2 (6) (7). Although no definite conclusions about the role of TRα2 can be drawn, both studies underline that the balance in TRα1:TRα2 may play an important role in the control of growth and homeostasis.

We show here that a high amount of T₃ decreases the endogenous TRα1:TRα2 ratio in HepG2 cells without affecting the transcription of the total TRα mRNA. This change in the TRα1:TRα2 mRNA ratio due to T₃ was also observed at the protein level. When HepG2 cells were exposed to sera from hyperthyroid patients we also found that high thyroid hormone concentrations decreased the TRα1:TRα2 mRNA ratio. Addition of T₃ to sera from hypothyroid patients similarly resulted in a decrease in the TRα1:TRα2 ratio. This change in the splicing direction towards the dominant negative receptor TRα2 could protect the (HepG2) cell against excessive T₃ induced gene expression, despite an extracellular environment containing high levels of T₃.

The mechanism behind this effect of T₃ is not clear. Unlike the gene encoding the TRβ isoforms, the gene encoding TRα does not contain a TRE and is therefore probably not under direct thyroid hormone control (23). Indeed, in our studies T₃ had no or only minor effects on transcription of the TRα gene, indicating that the effect of T₃ on TRα splicing is probably not regulated at the transcriptional level of the gene. Alternative splicing of TRα pre-mRNA could be regulated in various ways. One is the choice
of the alternative 5'-splice site, which is mediated by various splicing factors that bind to the alternative splice site but also to adjacent elements which results in expression of TRα1 or TRα2. In the intron of TRα2 a splicing enhancer element (SEα2) was characterized which in vitro stimulates TRα2 mRNA splicing. SEα2 binds SF2 which indicates that the splicing process might be influenced by binding of specific splicing proteins to this element (14;15;24). T₃ added at a concentration of 10⁻⁹M increased the hnRNP A1:SF2 ratio in HepG2 cells, which coincided with an increase in TRα2 expression. The TRα1:TRα2 ratio decreased although not significantly. This is in agreement with experiments that show that a predominance of SF2 over hnRNP A1 promotes the use of the proximal 5'-splice site, which in our case would lead to a high TRα1:TRα2 ratio. A high hnRNP A1:SF2 ratio results in the use of the distal 5'-splice sites, leading to a
The ratio of TRα1:TRα2 mRNA affects the splicing of TRα mRNA. It is known that both SF2 and hnRNP A1 can bind to specific RNA sequences and affect the splice site choice (12;13;25). Bai et al. have shown that co-transfection of SF2 with a calcitonin/CGRP gene construct promoted the use of the proximal 3'-splice site, resulting in the inclusion of a terminal calcitonin exon (20). Co-transfection with hnRNP A1 antagonized the effect of SF2 by stimulating the distal splice site. In our in vitro model only one alternative splice site is present, which is the distal splice site leading to TRα2 that is chosen over polyadenylation of TRα1. The discovery of the intronic enhancer element SEα2 within the final intron of TRα2 mRNA supports the idea that these splicing factors are involved and that the model proposed for calcitonin would be valid in this case as well (14). However, involvement of other, possibly tissue specific, splicing factors that play a role in the splicing process of the TRα pre-mRNA cannot be ruled out.

Regulation of the splicing process by thyroid hormone could also be mediated via signaling pathways leading to phosphorylation and subsequent activation of splicing factors.

Phosphorylation of SR proteins by CLK/Sty protein kinase leads to their release from nuclear speckles and enhances protein activity (26). Xiao and Manley showed that SF2 could only activate splicing in vitro when its serine residues in the RS domain are phosphorylated (27). It is possible that T₃ exerts its effect on splicing by serine phosphorylation of SF2. Since several kinases, such as CLK/Sty, SRPK1, PKA and PKC are all able to phosphorylate SF2, there are multiple candidates for regulation by T₃. Serine phosphorylation of TR isoforms itself has also been described. For example TRβ1 is stabilized by phosphorylation mediated by MAPKs via a nongenomic action of T₄ (28). Chen et al. have shown that MAPKs also potentiate TR activity. SF2, however is not a substrate for MAPK although other splicing factors might be involved that could be phosphorylated by MAPK. Jones et al. have shown a reduction in T₃-induced gene transcription as a result of a reduced TRα1 and TRβ1 activity due to the use of the serine/threonine kinase inhibitor H7 (29). In contrast to the effects observed on TR proteins by phosphorylation, no effects of phosphorylation have been described on TR isoform expression.

Another feature that could contribute to regulation of the TRα mRNA processing is RevErb. This anti-sense transcript encodes a nuclear receptor, belonging to the same superfamily as the thyroid hormone receptors. Only two natural target genes are known, the rat apolipoprotein (apo)A-1 gene and the human RevErb gene itself (30;31). Since the
RevErb mRNA partially overlaps the TRα2 mRNA, a possible role in direct regulation of the TRα gene expression by RevErb has been suggested. Transfection of a minigene expressing both TRα and RevErb genes, resulted in a 2-fold increase in the TRα1:TRα2 ratio as compared to TRα expression without co-expression of RevErb (32). A similar increase in the TRα1:TRα2 ratio and RevErb mRNA was found in adipocytes that were induced to differentiate in vitro (17). On the other hand, in a differentiating B-cell line, no correlation was found between the TRα1:TRα2 ratio and RevErb but a positive correlation was found between TRα1:TRα2 ratio and the RevErb:TRα2 ratio (33). In this case the authors in fact show a relation between TRα1 and RevErb, since both are divided by TRα2. In our study we did not find a correlation between RevErb and the TRα1:TRα2 ratio at all, suggesting that at least in this experiment, RevErb mRNA has no influence on the splicing of TRα pre-mRNA. The increased expression of RevErb in HepG2 cells incubated with NTI sera could be the result of low T3 contents in these sera since we also found a negative correlation between serum T3 and RevErb which is of unknown significance.

The low amount of T3 in NTI sera does not seem to have an effect on the splicing process of TRα pre-mRNA. IL-6 levels were elevated in serum of all NTI patients and correlated strongly with serum T3 which is in agreement with previous reports (34,35). IL-6 has been shown to contribute to the pathogenesis of NTI but the mechanism are not yet elucidated (36). In our experiment serum IL-6 was not related to the TRα1:TRα2 ratio but we cannot rule out the possibility that IL-6, via co-activators or splicing factors, plays an indirect role in the regulation of the splicing process. HepG2 cells incubated with NTI sera showed a relationship between a high hnRNP A1: SF2 ratio and a low TRα1:TRα2 ratio which is not related to serum T3. However this result supports the idea that a certain balance in the splicing factors hnRNP A1 to SF2 is involved in the splicing of TRα.

In conclusion, we have demonstrated that high amounts of T3 in incubation medium of HepG2 cells, either present in serum or supplemented, leads to a shift in the splicing direction of the endogenous TRα pre-mRNA towards the dominant negative isoform TRα2. This suggests a model where the change in splicing direction protects cells against excessive thyroid hormone regulated gene expression. In vivo this might provide a mechanism to keep tissues relatively euthyroid despite high serum T3 levels.
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