Alternative splicing of thyroid hormone receptor alpha transcripts during health and disease
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Thyroid hormone receptor alpha splice variants in livers of critically ill patients

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Abstract

Many physiological systems adapt to a changing (hormonal) environment by varying the levels of splice variants of a gene. An example is the c-erbA-α gene which through alternative splicing gives rise to two receptor isoforms TRα1 and TRα2. The TRα1 isoform is a bona fide thyroid hormone (T₃) receptor whereas the TRα2 acts as a dominant negative isoform. The ratio of these splice variants could therefore have a marked influence on T₃-regulated gene expression, especially in view of the changing metabolism of thyroid hormone during illness. We studied the TRα1:TRα2 ratio in postmortem liver biopsies of 58 patients who were critically ill and died in the ICU in relation to different clinical and treatment parameters. Stepwise multiple regression yielded the following equation: TRα1:TRα2 ratio = -1.854 + (0.0323*Age) + (0.0431*TISS score) indicating that 28% of the change in TRα1:TRα2 ratio can be predicted by these two clinical variables. There was no effect of randomization to intensive insulin therapy, glucocorticoid or thyroid hormone treatment on the TRα1:TRα2 ratio. The ratio hnRNPA1:SF2, a possible molecular determinant of the splicing ratio, did not show an association with the TRα1:TRα2 ratio.

In conclusion, it appears that in critically ill patients the ratio of TRα1:TRα2 expression is increased possibly indicating an increased sensitivity to T₃ but, in view of the decreased serum T₃ levels and the resulting possible suppression of T₃-responsive gene expression by unliganded TRs, the functional consequence of this increased ratio remains to be established.

Introduction

Many physiological systems adapt to a changing (hormonal) environment by varying the levels of splice variants of a particular gene (1). Examples include the splicing of glucose-6-phosphate dehydrogenase which changes as a result of nutritional status (2) or the change in splicing of the PKCβ gene induced by insulin (3). The precise mechanisms guiding these changes are not yet known but they are most probably caused by changing levels of splice factors. Two classes of proteins are known to be involved in splicing namely the hnRNP and the SR proteins (4). These proteins bind the RNA on specific sequences and thereby regulate the splicing direction. This direction is determined by the
ratio hnRNP/SR proteins where a high ratio favours the distal splice site whereas a low ratio favours the proximal one (5).

Splice variants are not only known for genes influenced by hormones but also for the hormone receptor genes themselves. One example is the dexamethasone dependent alternative splicing of the insulin receptor pre-mRNA which influences the sensitivity of a cell for insulin (6). Another example is the c-erbA-α gene which through alternative splicing gives rise to two receptor isoforms TRα1 and TRα2. Recent studies have indicated that the 9th exon of the c-erb-Aα gene contains a splice enhancer which can bind the above mentioned splice factors, which then determine the choice between TRα1 or TRα2 (7). The TRα1 isoform is a bona fide thyroid hormone receptor whereas the TRα2 is a non-binding form which is able to act as a dominant negative on the other receptor isoforms. The ratio of these splice variants could therefore have a marked influence on T₃-regulated gene expression. This idea is supported by recent data obtained from studies with knock-out animals lacking the TRα2 isoform which appeared to be more sensitive to the hormone (8;9). This finding can be interpreted as suggesting that the sensitivity of a particular cell/tissue for thyroid hormone may depend on the ratio of the α1 and α2 isoforms.

A major adaptation which occurs during illness or fasting is the changing metabolism of thyroid hormone. This phenomenon, termed non-thyroidal illness, causes a decrease in serum T₃ levels as a result of decreased membrane transport (10) and deiodination (11;12). Non-thyroidal illness can be viewed as an adaptation of the body to disease and the decrease in serum T₃ as a way to dampen catabolism. Although its cause and exact purpose remain elusive, especially during acute illness, it has been shown that in prolonged critical illness the suppressed pulsatile TSH release, possibly as a result of decreased hypothalamic TRH expression, accounts for much of the observed T₃ and T₄ decrease (13). If the underlying aim of non-thyroidal illness is to decrease the tissues sensitivity for T₃ then this could also be obtained by decreasing the activity of the receptor. Studies in mice have indeed shown that the receptor activity decreases during illness (14). On the other hand it could also be that the receptor becomes more sensitive in order to compensate for the decreased hormone availability.

In order to shed more light on these two possibilities we decided to study TR expression and the balance of TRα1:TRα2 in postmortem liver biopsies of 58 patients who were critically ill and died in the ICU (12). We evaluate the effects of patient characteristics like age and the severity of disease as well as hormonal interventions including...
intensive insulin therapy on the TRα1:TRα2 ratio. This ratio can be viewed as a marker for thyroid hormone sensitivity. Molecular determinants, such as SF2 and hnRNP A1 are studied for their possible involvement in the splicing process of the TRα pre-mRNA leading to alterations in the TRα1:TRα2 ratio.

Methods

Subjects

In this study we included 58 adult patients who participated in a large study (n=1548) on intensive insulin treatment in ICU patients of which the major clinical outcomes have been published elsewhere (15). On admission at the ICU, patients were randomized to conventional insulin therapy (maintenance of blood glucose level between 180 and 200 mg/dl, insulin started when blood glucose levels exceeded 210 mg/dl) or intensive insulin therapy (insulin infusion to maintain normoglycemia of 80-110 mg/dl, started when blood glucose levels exceeded 110 mg/dl). At the time of admission to the ICU, the severity of illness was determined by calculating the scores for the Acute Physiologic and Chronic Health Evaluation (APACHE II) (16) and the simplified Therapeutic Intervention Scoring System (TISS-28) (17;18). Higher scores indicate more severe illness and the requirement for more therapeutic interventions respectively. For the TISS-28 score, each therapeutic intervention is scored 1 to 4 points. Some patients (n=21) also received thyroid hormone treatment in the presence of clinical signs of hypothyroidism or serum T4 levels lower than 50 nmol/l with a normal thyroxine-binding globulin. Treatment consisted of an intravenous bolus of 150 μg T4 daily plus 0.6 μg T3 per kg bodyweight per 24h as a continuous IV infusion. All patients in this study have died in the ICU. Within minutes after death, blood samples were obtained from 43 patients and liver biopsies were obtained from 58 patients. This study protocol has been approved by the Ethical Review Board of the University of Leuven School of Medicine, and patients were included after informed consent from the closest family member.

Assays

The serum concentrations of TSH, T4 and T3 were measured by chemoluminescence assays (Vitros ECI Immunodiagnostic System, Ortho-Clinical Diagnostics, Amersham, UK). The within-assay coefficients of variation were 4%, 2% and 2% for TSH, T4 and
T₃ respectively. Reverse T₃ was measured by radioimmunoassay as previously described with a CV of 3-4% (19).

**RNA isolation and cDNA synthesis**

RNA was isolated from liver samples using the High Pure RNA Tissue Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. The RNA concentration in each sample was determined with the RiboGreen RNA Quantitation Kit (Molecular Probes, Leiden, The Netherlands) and all samples were diluted subsequently to 0.1 μg/μl. Single-stranded cDNA was obtained using 1μg of RNA and the First Strand cDNA synthesis kit with random primers (Roche Molecular Biochemicals, Mannheim, Germany).

**Real-time PCR**

Real-time PCR reactions were performed in a LightCycler (Roche Molecular Biochemicals, Mannheim, Germany). TRα1 (forward, 5'-CATCTTTGAACGTGGGCAAGT-3'；reverse 5'-CTGAGGCTTTAGACTTCTCAGT-3') and TRα2 (forward, 5'-CATCTTTGAACTGGGCAAGT-3'；reverse 5'-GACCCTGAACACATTCAGT-3') were simultaneously detected (dual colour detection) in the same sample using sequence-specific hybridization probes (TRα1 forward probe, 5'-GGCCCAAGCTGCTGATGATG-Fluorescein-3'; TRα1 reverse probe 5'-LCred640-TGACTGACCTCCGATCATCG-P-3'; TRα2 forward probe, 5'-GGCCCAAGCTGCTGATGATG-Fluorescein-3'; TRα2 reverse probe 5'-LCred704-GAGTTGAGTACCGTATGCTGAT-3'; manufactured by TIB-MolBiol, Berlin, Germany) and the LightCycler-FastStart DNA Master Hybridization Probes kit (Roche Molecular Biochemicals, Mannheim, Germany). Further details were as previously described (20).

SF2 and hnRNP A1 were measured in a total reaction volume of 20 μl with 2μl of cDNA using the LightCycler-DNA Master SYBR Green kit. The sequences of the primers of SF2 and hnRNP A1 were as described (21): For each mRNA assayed, a sequence-specific standard was generated and analyzed in the range of 0.1-1000 fg/20 μl in parallel to the samples. The crossing points of the standards with the noise band, which is set at the beginning of the log-linear phase, are plotted against the logarithm of the concentration and fitted to a standard curve. The concentration of cDNA of each gene is then calculated from its own standard curve. Since only ratios of mRNA are described in this paper, there was no need to correct for a housekeeping gene.
Chapter 3

Statistical analysis

Correlations between data were analyzed using Pearson’s correlation coefficient for the whole group and for subgroups Mann-Whitney U tests were used. To identify possible determinants of the TRα1:TRα2 ratio among the different clinical parameters we used stepwise multiple regression. We used SPSS 10.0.7 (SPSS, Chicago, IL) for all analyses.

Results

Baseline characteristics of patients are shown in table 1. Severity of illness was determined during the first 24 hours in the intensive care unit (ICU) by calculating the scores for the APACHE II. Higher scores indicate a more severe illness. The simplified Therapeutic Intervention Scoring System (TISS-28) reflects the total score of therapeutic interventions that were done on each patient during the first 24 hours. Serum concentrations of thyroid hormones are given in table 2.

Table 1. Characteristics of 58 patients admitted to the ICU with critical illness who subsequently died.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male sex</td>
<td>36 (62%)</td>
</tr>
<tr>
<td>Age - yr (mean ± SD)</td>
<td>70.7 ± 11.7</td>
</tr>
<tr>
<td>Body-mass index (mean ± SD)</td>
<td>25.6 ± 4.8</td>
</tr>
<tr>
<td>Hormonal intervention</td>
<td>42 (72%)</td>
</tr>
<tr>
<td>Intensive insulin therapy</td>
<td>20 (35%)</td>
</tr>
<tr>
<td>Thyroid hormone treatment</td>
<td>21 (36%)</td>
</tr>
<tr>
<td>Glucocorticoid treatment</td>
<td>29 (50%)</td>
</tr>
<tr>
<td>APACHE II score (median and range)</td>
<td>13 (4-41)</td>
</tr>
<tr>
<td>TISS-28 score (median and range)</td>
<td>40 (17-55)</td>
</tr>
<tr>
<td>Renal Replacement therapy</td>
<td>26 (45%)</td>
</tr>
<tr>
<td>ICU stay - days (median and range)</td>
<td>11 (1-10)</td>
</tr>
</tbody>
</table>

Patients who were treated with thyroid hormones had significantly lower TSH levels and higher T₃ levels as well as higher T₃/T₄ ratios compared to patients who were not treated.
Figure 1: A. Correlation of the TRα1:TRα2 mRNA ratio in liver biopsies of 58 patients who died in the ICU and their age. B. Correlation of the TRα1:TRα2 ratio with the amount of therapeutic interventions that a patient received during the first 24 hours in the ICU (TISS score day 1). C. Correlation of the TRα1:TRα2 ratio and the APACHE II score during the first 24 hours in the ICU.

Using the dual colour detection option on the LightCycler we were able to measure the TRα1:TRα2 ratio in each individual sample without having to correct for input, efficiency of the sample etc. The conditions were chosen such that the slopes of the standard curves for the two TR isoform mRNAs (indicative of the PCR efficiency) were analogous (Δslope<0.05), thus making sure that a reliable quantification could be obtained over a large range of concentrations.

The TRα1:TRα2 mRNA ratio was calculated in all liver biopsies and we found that it significantly correlated with age, TISS score on day 1 and APACHE II (Fig. 1). The correlation with age remains significant when the subjects in the 20-40 years age range
Table 2. Serum thyroid hormone parameters in the total group (n=43) and subdivided in a group not receiving thyroid hormone (TH) treatment (n=28) and a group receiving TH treatment (n=15).

<table>
<thead>
<tr>
<th>Normal values</th>
<th>No TH treatment</th>
<th>TH treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSH mU/l</td>
<td>0.2-4.2</td>
<td>0.63 (0.02-4.5)</td>
</tr>
<tr>
<td>T&lt;sub&gt;4&lt;/sub&gt; nmol/l</td>
<td>58-128</td>
<td>27.6 (5.4-121.0)</td>
</tr>
<tr>
<td>T&lt;sub&gt;3&lt;/sub&gt; nmol/l</td>
<td>1.43-2.51</td>
<td>0.78 (0.41-4.71)</td>
</tr>
<tr>
<td>rT&lt;sub&gt;3&lt;/sub&gt; nmol/l</td>
<td>0.14-0.34</td>
<td>0.84 (0.24-15.78)</td>
</tr>
<tr>
<td>T&lt;sub&gt;3&lt;/sub&gt;/rT&lt;sub&gt;3&lt;/sub&gt; molar ratio</td>
<td>4.2-17.3</td>
<td>0.85 (0.18-6.13)</td>
</tr>
<tr>
<td>T&lt;sub&gt;3&lt;/sub&gt;/T&lt;sub&gt;4&lt;/sub&gt; molar ratio</td>
<td>0.01-0.04</td>
<td>0.02 (0.013-0.089)</td>
</tr>
</tbody>
</table>

Median and range is given.
** p<0.001 and * p<0.05 vs not treated patients

were removed. We also found a significant difference in the TRα1:TRα2 ratio as a result of inotrope and renal replacement therapy (RRT) (Fig. 2). Other variables which were tested but which showed no significant relation to the TRα1:TRα2 ratio were: peak serum values of urea, creatinine and C-reactive protein and the mean morning blood glucose level.

Figure 2: Box-and-whisker plots of the TRα1:TRα2 mRNA ratio in liver biopsies of 58 patients who died in the ICU divided by treatment with inotropes (2A) or renal replacement therapy (RRT, 2B).
Figure 3: Box-and-whisker plots of the TRα1:TRα2 mRNA ratio in liver biopsies of 58 patients who died in the ICU divided by randomization to conventional or intensive insulin therapy (3A), treatment with thyroid hormones (TH, 3B) or treatment with glucocorticoids (GC, 3C).

The clinical variables that correlated to the TRα1:TRα2 ratio with a p-value less than or equal to 0.05 were used in a multiple regression analysis which yielded the following equation: TRα1:TRα2 ratio = -1.854 + (0.0323*Age) + (0.0431*TISS score). This indicates that 28% of the change in TRα1:TRα2 ratio can be predicted by these two variables. No significant contribution to this model was found from the other variables.

There was no effect of the randomization to either conventional or intensive insulin therapy on the TRα1:TRα2 ratio (Fig. 3A). We next looked at the effect of thyroid hormone treatment or glucocorticoid treatment and again no difference was found in the TRα1:TRα2 ratio between the treatment groups (Fig 3B and 3C).
In view of the wide variation in the TRα1:TRα2 ratio we looked whether there is a relation between the etiology of disease or the cause of death and the level of the ratio. To this end we grouped the patients according to the etiology or cause of death and checked whether the extremes (defined as those values in the first or the last quartile) of the ratio could be explained by one or more of the subclasses, but no significant relation was found (table 3).

The serum $T_3/rT_3$ ratio correlated negatively with the TRα1:TRα2 ratio ($p<0.05$) (Fig 4) but the ratio hnRNPA1: SF2, a possible molecular determinant of the splicing ratio, did not show an association with the TRα1:TRα2 ratio (Fig 5).

**Table 3.** Cause of illness and death in all patients and number of patients in the 1st and 4th quartiles of the TRα1: TRα2 ratio.

<table>
<thead>
<tr>
<th>Pathology</th>
<th>1st quartile (ratio&lt;1.39)</th>
<th>4th quartile (ratio&gt;2.53)</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiac</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Vascular</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Thoracic</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Transplant</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Abdominal</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Cerebral</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Polytraumatic</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Diverse</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Cause of death</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiac shock</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>MOF sepsis</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>MOF SIRS</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Severe brain damage</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

MOF: multiple organ failure.; SIRS: systemic inflammatory response syndrome

**Discussion**

Several non-thyroid dependent conditions are known in which plasma $T_3$ levels decrease and in which one could expect adaptations in the sensitivity of the receptor towards hormone, in particular illness and increasing age. Many publications deal with the decrease of $T_3$ in the blood as a result of illness. It has been argued that the illness-
induced decrease in T₃ is an adaptation of the system to the diseased state such that it will decrease the impact of T₃ on its target organs ((22) and references therein). However, studies in prolonged critically ill patients have shown that, even though they have severely decreased thyroid hormone levels, they are in fact in a state of catabolism (23). In general the decrease in T₃ levels is more pronounced as the illness gets worse. Initially the T₃ decrease is not accompanied by changes in TSH levels but these do change as the illness lasts. The TSH decrease is a result of a decrease in TSH pulse amplitude and it has been shown that this suppressed pulsatile TSH release accounts for much of the observed T₃ and T₄ decrease in prolonged critical illness (13). On top of that, further studies have shown that the decreased tissue levels of deiodinase I play an important role in the decreased formation of the active hormone T₃ and, together with an increase in deiodinase III activity in both liver and muscle, contribute to the changes seen during critical illness (12). A recent publication on the relation between T₃ and age showed a decreased FT₃/FT₄ ratio in old and very old (centenarians) humans (24). Again, as in the case of illness, it is suggested that changes in the activity of the deiodinases are at the root of the decreasing ratio.

Theoretically, tissues may adapt to the decrease in circulating hormone by increasing the sensitivity for the hormone, e.g. by modulating the number of receptors. Interestingly, two of the parameters that showed a correlation to the TRα1:TRα2 ratio in our study were age and severity of illness (expressed as APACHE II). In both cases a positive correlation was found, indicating that the system tries to adapt to the decreasing T₃ by increasing the amount of active receptor over the inactive - dominant negative – one,

**Figure 4:** Correlation of the TRα1:TRα2 ratio in liver biopsies of 43 patients who died in the ICU and their serum ratio T3/T3.

**Figure 5:** Correlation of the TRα1:TRα2 ratio and the ratio of splicing factors hnRNP A1:SF2 in mRNA samples of 58 liver biopsies of patients who died in the ICU.
which is supported by the negative relationship between the TRα1:TRα2 ratio and T₃/rT₃ ratio (as an indirect measure of the severity of illness). This increased tissue sensitivity could give rise to a catabolic state which is indeed what has been observed in critically ill patients (23).

The increase in the TRα1:TRα2 ratio is not dependent on changes in the expression of the splice factors SF2 and hnRNPAl since we did not find a relation between the ratio of these splice factors which is known to determine the splice site selection (5). We cannot rule out however that these proteins do play a role because we only measured their mRNAs. It is known that the activity of these proteins is influenced by their phosphorylation state and this is therefore possibly of more importance (25). We have not been able to measure this since only RNA was available from the small amounts of tissue biopsies. In a previous study in HepG2 cells incubated with sera from NTI patients we did find a relation between the TRα1:TRα2 ratio and the ratio of the splicing factors (21) which however was independent of the serum T₃ level. This difference may be explained by the fact that the patient group in the present study is biased towards the more severely ill than that in our earlier study which included sera from patients across a wide spectrum of NTI and healthy controls.

When looking at the different hormonal treatments we found no effect of these treatments on the TRα1:TRα2 ratio indicating that there is no direct effect of these hormones on the splicing of the TRα pre-mRNA in this particular situation. In vitro we recently did find an effect of (pharmacological doses of) thyroid hormone on the splicing of the TRα pre-mRNA (21). That we have not found an effect of thyroid hormone in this study with critically ill patients may be due to the fact that the thyroid hormone treatment in these patients was aimed at restoring euthyroidism. Moreover, the effect of thyroid hormone on the splicing direction may be overruled by the (stronger) effects of the illness. The results presented here also differ from those obtained after fasting in rats where the TRα1:TRα2 ratio decreased (26), indicating that the NTI induced by critical illness has a different underlying mechanism (cytokines?) than that of fasting.

Concluding it appears that, in contrast to the suggestion that T₃ decreases during NTI to dampen T₃ action, the system tries to adapt to the decreasing thyroid hormone levels during prolonged (critical) illness by increasing the expression of the active form of the thyroid hormone receptor α and thereby possibly increasing the cellular sensitivity to the hormone.
Reference List


