Understanding the non-thyroidal illness syndrome from in vivo and in vitro studies

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Chapter

General discussion
9 General discussion

The aim of this thesis was to study the mechanisms and cellular signaling pathways involved in the changes observed in thyroid hormone metabolism during illness by using both in vivo and in vitro models. Although altered TH secretion, transport and clearance are known to contribute to NTIS (1), in this thesis we have focused on the role of cytokines, TRs and deiodinases during NTIS.

9.1 Hypothalamus

In animal models of NTIS, the observed changes in hypothalamic thyroid hormone metabolism are remarkably similar. D2 expression increased (chapter 2) (2-4), specifically in the tanycytes lining the third ventricle (5;7), while D3 expression decreased in the periventricular area (PE) after LPS administration (4) and during turpentine induced chronic inflammation (2). The change in D3 expression was not observed when the hypothalamus was processed entirely in critically ill rabbits (7). As proposed by Lechan (8) and Fliers (9), the local upregulation of D2 and downregulation of D3 during illness probably results in increased local T3 production, which subsequently leads to decreased TRH expression in the PE as observed during illness in animals and in humans (2-5;7;10). At variance with this hypothesis, hypothalamic T3 content was unchanged in critically ill rabbits despite increased D2 mRNA expression (7), but this might be due to the fact that changes in D2 and TRH expression occur in discrete hypothalamic areas including the PE and PVN, whereas the whole hypothalamus was processed for measuring T3 contents in the study reported by Mebis et al.

Hypothalamic TR mRNA expression did not change during illness (chapter 2) (7), but specific TR expression in the tanycytes or PE has not been evaluated during illness to date. As the increase of D2 and the decrease of D3 were more pronounced and the decrease of TRH was less pronounced in TRβ-/ mice (4), the TRβ appears to play a regulatory role in these changes.

The proposed mechanism of TRH suppression during illness is reminiscent of the TRH-suppression during fasting. Inhibition of fasting-induced D2 activity in the ARC blunts the fasting-induced TRH mRNA decline (11). In contrast to the fasting-induced upregulation of D2 which is related to the interplay between decreased serum leptin and increased serum corticosterone (12), the mechanism behind the hypothalamic D2 increase during illness is unclear at the moment. It has been reported that the hypothalamic D2 upregulation after LPS is independent of the decreased serum TH levels in rats (13). D2 might be upregulated during inflammation via activation of the NFkB pathway, as the D2 promoter contains NFkB responsive elements (5;14). However, as evident from longitudinal studies, hypothalamic D2 activation precedes hypothalamic NFkB upregulation, arguing against this notion (E. Sanchez et al., abstract Endocrine Society, 2007).
Chapter 9

9.2 Pituitary

One of the hallmarks of NTIS is the unresponsiveness of the pituitary to the low serum thyroid hormone levels, which means that the regular feedback mechanism of the HPT axis is disturbed during illness (1).

Indeed, during illness, decreased TSHβ mRNA or decreased serum TSH levels are observed in all studied animal models of NTIS (chapter 2) (2;4;7;15) (S.pneumoniae model: J. Kwakkel, unpublished data).

Both D1 and D2 are expressed in the anterior pituitary. The negative feedback of thyroid hormone on pituitary TSH has been proposed to be regulated via local D2-mediated conversion of T₄ into T₃, which is subsequently bound by the TRs, resulting in repression of the TSHβ gene (16).

The crucial role of pituitary D2 in TSH regulation is supported by the disturbed thyroid hormone-TSH feedback mechanism is D2- knockout mice (17). It is tempting to speculate that the observed downregulation of TSHβ during illness might be similar to the hypothalamic TRH repression during illness, resulting from increased D2 expression. However, in contrast to hypothalamic D2, pituitary D2

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**Figure 1.** Proposed model for illness-induced changes in thyroid hormone metabolism in the hypothalamic periventricular region. The 3rd ventricle (III) is shown in the middle, with on the left side the basal situation and on the right side the situation during inflammation: During inflammation D2 activity increases in the tanycytes lining the 3rd ventricle wall, while D3 activity decreases, presumably resulting in increased local T₃ concentrations, which ultimately lead to repression of the TRH gene in the PVN via TRβ.
expression varies during illness, depending on genetic background or type of illness (chapter 2) (4,5,15,18,19). Experiments in cytokine knock-out mice have shown that both the increase and decrease of D2 during illness is cytokine-independent (18,19). In addition, we observed decreased TSHβ mRNA expression after LPS administration in mice despite decreased D2 expression (chapter 2) (S. pneumoniae: J. Kwakkel, unpublished results). These observations exclude D2 as a contributor to altered TSH expression during illness, which is supported by a study of Wassen et al, who reported that the cytokine-induced TSH decrease was independent of nuclear T3 binding in a primary culture of anterior pituitary cells (20).

A possible mechanism of decreased pituitary TSH expression might be decreased pituitary TR expression during illness as we have reported in chapter 2. This is consistent with the less pronounced TSHβ decrease in TRβ−/− mice (4). However, more studies on TR expression and the mechanism of ligand-independent gene activation are needed. Alternatively, in vivo TSH suppression during illness might also be the result of decreased TRH release, as TRH is a strong regulator of TSH both on pre- and posttranscriptional level (21) and TRH mRNA expression is reduced in the PVN during illness (2-4,7,10). Alternatively, hypothalamic increase of D2 during illness might result in extra T3-release in the portal capillary system which might exert direct effects of TSH expression (22).

Pituitary D2 expression after LPS administration in rats increases, whereas in T4-clamped LPS-treated rats D2 expression decreases, indicating that the inhibitory effect of LPS on pituitary D2 expression is overridden by the stimulatory effect of the decreased serum TH levels after LPS administration (13).

The LPS-induced decrease of D1 is solely dependent on pro-inflammatory cytokines, as pituitary D1 decrease was abolished in mice devoid of IL-12, IL-18 and the IFNγR (18,19), but not in TRβ−/− mice (4). However, the function of pituitary D1, and the possible contribution of D1 to TSHβ expression remains to be elucidated.

9.3 Thyroid

Thyroidal TSH-R and D1 mRNA expression appeared to decrease after LPS administration in mice, while thyroidal TRα1, TRα2 and TRβ1 mRNA expression did not change (chapters 2 and 5). Decreased thyroidal release of 131I during pneumococcal infection was observed in mice and rats. However, although 131I uptake was decreased in rats during pneumococcal infection, in mice no difference was observed (23). These effects might be due to decreased stimulation of the thyroid by TSH, as serum TSH and thyroidal TSHR levels decrease during illness. However, our results in chapter 2 showed that acute alterations occur simultaneously in pituitary and thyroid indicating that other mechanism play a role in the early effects on the thyroid. This is supported by a study of De Jongh et al, who observed
reduced thyroid weight in chronically ill patients (>3 months), but not in a group of ICU patients with acute or subacute disease (24). The effects of pro-inflammatory cytokines (IL-1, IL-6 and TNFα) on thyrocytes have been studied extensively in vitro and are reported to decrease Tg synthesis (25;26), D1 (27;28), D2 (29) and TPO (30) expression, $^{125}$I incorporation (31) and T$_3$ release (31). It has been shown that altered mRNA stability and competitive inhibitors contribute to decreased thyroidal D1 activity during illness (32). It is however unknown which signal transduction pathways are involved. Based on these in vitro studies, we conclude that the early decrease of TH secretion might be primarily cytokine mediated. Surprisingly, in chapter 5 we observed that the decrease of serum TH was attenuated in TRα0/0 mice, suggesting involvement of TRα in the illness-induced alterations in either TH secretion or TH clearance. However, as thyroidal TRα1 and TRα2 mRNA expression did not change upon LPS administration and no aberrant thyroidal histological phenotype has been reported for TRα0/0 mice, the involvement of TRα in TH secretion seems unlikely.

9.4 Liver
The liver is an important organ during illness, as it orchestrates the production of acute phase proteins (APPs). Liver is also an important target organ for TH as TH influences liver glucose and lipid metabolism and mitochondrial activity. In liver TRβ1, TRα1 and TRα2 isoforms are abundantly expressed, and it is reported that approximately 60% of liver T$_3$-regulated genes are TRβ-dependent (33). D1 and D3 are both expressed in liver, although D3 is expressed at very low levels during normal circumstances. As D1 is a T$_3$-regulated gene (34;35), we evaluated not only deiodinase expression, but also liver TR expression during illness.

**D1:** During illness, liver D1 expression decreases and it is generally thought that the D1-decrease contributes significantly to the low serum T$_3$ levels observed during illness. Indeed, in chapter 2 we observed decreased liver D1 expression after LPS administration, followed by decreased serum T$_3$ levels (chapter 2). Liver D1 regulation is assumed to be primarily driven via the TRβ (36;37), but to our surprise we observed that during illness the downregulation of D1 is partly mediated via TRα, not via TRβ (chapters 4 and 5).

These in vivo results are supported by our in vitro studies in HepG2 cells described in chapter 6 and 7. The IL-1β-induced decrease of TRβ mRNA was mediated by the NFκB pathway solely, while the IL-1β–induced decrease of D1 and TRα mRNA was abolished by simultaneous inhibition of NFκB and AP-1. The cytokine-induced D1 decrease is due to inhibition of D1-promoter activity (38), which we now assume to be partly mediated via TRα.
As the D1-gene is activated via a TR/RXR heterodimer, decreased D1 expression might also be due to decreased nuclear RXRα protein. Nuclear RXR protein decreases after LPS administration, due to rapid nuclear export via JNK-phosphorylation and subsequent proteasomal degradation (39-41). However, since the IL-1β-induced decrease of D1 mRNA was not prevented by inhibition of JNK alone as demonstrated in chapter 6, we postulate that decreased RXR expression is not predominantly involved in the D1 mRNA decrease observed during illness. Another mechanism has been proposed by Yu et al, who have shown both in vivo and in vitro that adding exogenous co-activator SRC-1 attenuated the illness-induced liver D1 decrease (42;43). These studies indicate that competition for limiting amounts of SRC-1, which is a shared coactivator for both TR and inflammatory signaling pathways, is one of the mechanisms involved in the illness-induced D1 decrease. Moreover, restoration of liver D1 expression by exogenous SRC-1 prevented the development of NTIS after LPS administration, pointing to liver D1 as a sole contributor to decreased serum T3 levels after LPS administration (43). In contrast, in the burn-injury rabbit model of prolonged illness the D1 decrease might be mediated via decreased T3 levels, as liver D1 is associated with serum T3 levels and infusion of T4 and T3 abolishes the liver D1 decrease (44). The role of T3 in the illness induced D1 decrease remains controversial, as we have observed LPS-induced liver D1 decrease to be preceded by serum T3 decrease (chapter 2) and the opposite as shown in chapter 4.

**D3:** Increased liver D3 expression has also been postulated as a possible contributor to decreased serum T3 levels during prolonged critical illness in humans (44;45). The D3 increase is proposed to be due to tissue hypoxia (45), mediated via the transcription factor hypoxia inducible factor (HIF)-1α (46). Surprisingly however, we observed decreased liver D3 expression after LPS administration and during turpentine-induced chronic inflammation (47). In chapter 5 we reported that the LPS induced liver D3 decrease was abolished in TRβ-/- mice and aggravated in TRα0/0 mice. Because TRα0/0 mice have more sensitive TRβ signaling (37), our results indicate that the LPS-induced D3 decrease is mediated via TRβ. More importantly, serum TH levels were not different between TRβ-/- and WT mice, suggesting that decreased liver D3 does not contribute to changes in serum TH hormone levels after LPS administration. This is supported by a recent study in D3KO mice, wherein we reported a similar decrease of serum TH levels in S. pneumoniae infected D3KO mice compared to WT mice (48).

**TRs:** Liver TRα1, TRα2 and TRβ1 mRNA mRNA decreased rapidly upon LPS administration (chapters 2 and 7) (49), followed by decreased nuclear TR protein expression 16h after LPS administration (49). In chapter 7 we reported that the IL-1β-induced decrease of TRα1 and TRα2 is a direct effect of decreased
promoter activity. The exact mechanism remains unknown, but might involve phosphorylation-dependent repression of the TRα promoter. In contrast to TRα, the IL-1β-induced decrease of TRβ1 is mediated via the NFκB pathway solely and is partly due to decreased promoter activity and decreased mRNA stability (J. Kwakkel, unpublished data). *In silico* analysis (50) revealed the presence of three
NFκB responsive elements in the TRβ-promoter, which might be involved in the NFκB dependent repression of the TRβ gene.

9.5 Skeletal muscle

As it had been reported that skeletal muscle D2 is involved in the peripheral production of T₃ under normal circumstances (51), we evaluated in chapter 3 and 4 whether decreased muscle D2 expression contributes to the low serum T₃ levels observed during illness. In contrast to our hypothesis, we observed increased muscle D2 expression after LPS administration and during turpentine induced chronic inflammation (chapters 3 and 4). The illness-induced increase of muscle D2 is in line with increased D2 expression observed in skeletal muscle of ICU patients (52) and suggests that muscle D2 might be more important in local T₃ generation rather than contributing to serum T₃ levels.

During S.pneumoniae infection however, muscle D2 expression decreased, which corresponds with previously reported D2 decrease in muscle tissue of septic patients (53). We cannot exclude the effect of fasting in decreased D2 expression during illness, as D2 expression decreased after fasting in healthy humans (54). In addition, D2 mRNA expression also decreased in the pair-fed controls of the chronically inflamed mice (chapter 3).

We also evaluated muscle D3 expression in different animal models of illness. After LPS administration D3 expression decreased (chapter 5), whereas during S.pneumoniae infection D3 expression was unchanged (chapter 3). Unexpectedly, during turpentine induced chronic inflammation D3 expression increased simultaneous with D2 expression (chapter 3). Although increased D3 expression had been previously reported (53), the simultaneous increase of D2 and D3 in the same tissue had not been reported before. The differential regulation of muscle D2 and D3 expression theoretically will have a different outcome with regard to local T₃ and T₂ concentrations as depicted schematically in figure 3. Both T₃ and T₂ are known to be regulators of metabolic state (55;56). We hypothesize that differential regulation of deiodinase expression during illness might be relevant for regulating changes in muscle metabolic state during different stages of disease.

We conclude that changes in muscle deiodinase expression are dependent on type and severity of illness and are likely regulated via different mechanisms. In chapter 3 we associated D2 expression in muscle during illness with known D2-regulating factors. We found no overall association with serum thyroid hormone levels or D2-ubiquitinating factors. However, activation of the cAMP pathway coincided with the simultaneous induction of D2 and D3 in muscle tissue of chronically inflamed mice, which has been confirmed by in vitro experiments as described in chapter 8. We did not observe a consistent association with proinflammatory cytokines or
the activation of inflammatory pathways (chapter 3). This is supported by our in vitro observation that stimulation of myoblasts and myotubes with inflammatory mediators like cytokines or LPS did not alter D2 or D3 expression (chapter 8).

The LPS-induced decrease of muscle D3 was less pronounced in TRα0/0 mice, indicating involvement of TRα or serum thyroid hormone levels, which decrease to a lesser extent in TRα0/0 after LPS administration. The regulation of muscle D3 via TRα is supported by lower basal muscle D3 levels in TRα0/0 mice but at variance with the observation that TRα expression decreased only in the forelimb tissue upon LPS administration, while the effects on D3 are in both fore- and hindlimb muscle (chapter 5). In contrast, the LPS-induced increase of D2 was more pronounced in TRβ-/- mice (chapter 4), suggesting that TRβ partly suppresses D2 induction during illness. However, we observed no differences in the TRα0/0 mice (chapter 5), which have a more sensitive nuclear TRβ signalling (37). This phenomenon might be due to the either the low expression level of TRβ in muscle tissue or a non-genomic action of TRβ; alternatively, it might a secondary effect to the deletion of the TRβ gene.

**Figure 3.** Schematic representation of the alterations in muscle deiodinase expression in acute inflammation (LPS administration, upper panel), chronic inflammation (turpentine induced abscess, middle panel) and severe bacterial infection (*S. pneumoniae* infection, lower panel). On the left side the observed alterations in deiodinase expression, on the right side the theoretical net result on local T3 and T2 concentrations.
9.6 Concluding remarks

In this thesis we aimed to gain insight in the molecular changes in thyroid hormone metabolism during illness, focussing on TRs and deiodinases. We showed that these changes largely depend on type and severity of illness. The illness-induced changes in the hypothalamus were similar in all animal models of illness studied, whereas changes in pituitary and peripheral tissues appeared to differ between various experimental animal models. Furthermore, the acute changes occurred simultaneously in a variety of organs and therefore appear to be independent of each other.

Cytokines and the activation of the inflammatory pathways play an important role in the illness-induced changes observed in liver. From our studies in mice devoid of TRs we conclude that the changes in the central part of the HPT-axis are predominantly mediated via TRβ, whereas the peripheral changes in serum, liver and muscle appear to be partly dependent on TRα, with the exception of the illness-induced liver D3 decrease, which is regulated via TRβ (figure 4). Ending up with a variety of mechanisms that might be involved in altered TH metabolism, the question remains how serum thyroid hormone levels decrease during illness.

As mice devoid of deiodinases display no differences in basal serum T3 levels, the contribution of illness-induced changes in deiodinase expression to serum TH levels is presently unclear.

Decreased release of TH from the thyroid and/or increased TH-clearance might be more important than altered deiodinase activities in the rapid decrease in serum TH levels during illness and needs to be studied further in the future.

Although the contribution of the thyroid gland in serum T3 production differs between humans and rodents (20% in humans versus 50% in rodents), suggestive of a more prominent role for the deiodinases in humans compared to rodents, our results obtained in experimental models are largely in agreement with the limited amount of results obtained in human tissues.

The role of thyroid hormone transporters during illness has not been taken into account in this thesis. Thyroid hormone transport is an important factor in thyroid hormone metabolism and is known to be reduced by serum-factors of NTIS patients in vitro in hepatocytes (57). In critically ill rabbits, liver MCT8 and muscle MCT10 expression are upregulated presumably as a compensatory mechanism for the low serum TH levels (58). However, in a study in post-mortem tissue of ICU patients no correlation between serum TH levels and liver and muscle MCT8 expression was observed (59). The role of thyroid hormone transporters and the effect on the tissue TH levels in our animal models of illness remains to be studied.

We propose that the changes in deiodinase expression in TH target tissues contribute to changes in local tissue concentrations of TH and its derivatives, which are known regulators of tissue metabolic state.
During illness energy demands change dramatically, since activating the immune system costs energy while food-intake is usually decreased. The finding that TRα, an important metabolic regulator, is involved in the peripheral changes of NTIS supports the notion that NTIS mediates a change in tissue metabolic state in response to illness. However, more research is needed to 1) gain further insight into liver and muscle metabolic state during different stages and types of illness, 2) determine to what extent the deiodinases contribute to the changes in metabolic state and 3) establish whether these changes are adaptive or maladaptive, as it has been proposed that during the acute stage of illness NTIS is an adaptive response to support the immune response, while during protracted chronic illness NTIS is probably maladaptive as the body is being kept alive artificially (60).
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