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

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Simultaneous changes in central and peripheral components of the hypothalamus-pituitary-thyroid axis in lipopolysaccharide (LPS)-induced acute illness in mice

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Abstract

During illness, major changes in thyroid hormone metabolism and regulation occur, collectively known as the nonthyroidal illness syndrome (NTIS) and characterised by decreased serum T₃ and T₄ without an increase of serum TSH. Whether alterations in the central part of the hypothalamus-pituitary-thyroid (HPT)-axis precede changes in peripheral thyroid hormone metabolism instead of *vice versa*, or occur simultaneously is presently unknown. Therefore we studied the time course of changes in thyroid hormone metabolism in HPT-axis of mice during acute illness induced by bacterial endotoxin (LPS).

LPS rapidly induced IL-1β mRNA expression in hypothalamus, pituitary, thyroid and liver. This was followed by almost simultaneous changes in pituitary (decreased expression of TRβ2, TSHβ, and D1 mRNA’s), thyroid (decreased TSH-R mRNA) and liver (decreased TRβ1 and D1 mRNA). In the hypothalamus D2 mRNA expression was strongly increased whereas preproTRH mRNA expression did not change after LPS. Serum T₃ and T₄ fall only after 24 hrs.

Our results suggest almost simultaneous involvement of the whole HPT-axis in the down-regulation of thyroid hormone metabolism during acute illness.
Introduction

During illness, profound changes in thyroid hormone metabolism and regulation occur, collectively known as the nonthyroidal illness syndrome (NTIS) or “sick euthyroid syndrome” (SES). These changes include decreased serum T₃ and T₄ levels and increased serum rT₃ levels. Despite low serum thyroid hormone levels, serum TSH does not increase and can actually be decreased. Several mechanisms are involved in the alterations in thyroid hormone metabolism: decreased TRH expression in the hypothalamus, decreased thyroid hormone release by the thyroid gland, decreased transport of thyroid hormones across the plasma membrane, and a decrease in extrathyroidal (peripheral) conversion of T₄ into T₃ by 5’-deiodinase (D1), notably in the liver (Wiersinga 2000).

It has been hypothesized that during the acute phase of illness, changes in thyroid hormone metabolism are predominantly caused by peripheral adaptations while anterior pituitary function is still unaltered. In prolonged critical illness however, down-regulation of the central part of the hypothalamus-pituitary-thyroid-axis (HPT-axis) plays an important role (Van den Berghè). TSH secretion by the anterior pituitary decreases probably as a result of diminished hypothalamic stimulation as evident from decreased TRH gene expression in the paraventricular nucleus (PVN) of deceased patients with documented nonthyroidal illness (Fliers et al. 1997). In addition, the combined administration of TRH and GHRP-2 (growth hormone releasing factor-2) enhances pulsatile TSH secretion dramatically in intensive care patients under restoration of plasma T₄ and T₃ levels, in keeping with an important role for the hypothalamus in the central down-regulation of the HPT axis in prolonged illness (Van den et al. Berghè).

Few animal experimental data are available of the effects of acute illness on the central part of the HPT-axis. Intraperitoneal administration of bacterial endotoxin (LPS) resulted in decreased serum T₃ and T₄ levels after 24 h and inappropriately normal or low proTRH mRNA content in the paraventricular nucleus (PVN) of rats (Kakucska et al. 1994). Recently, we showed that LPS administration in mice results in a rapid decrease of type 2 deiodinase (D2) activity in the pituitary (Boelen et al. 2004) indicating an early response of the pituitary during acute illness.

Whether alterations of the central part of the HPT-axis precede changes in peripheral thyroid hormone metabolism instead of vice versa, or occur simultaneously, is presently unknown. Therefore we studied the time course of changes in thyroid hormone metabolism, characterized by mRNA expression of thyroid hormone related genes in the hypothalamus, pituitary, thyroid and liver of mice during acute illness induced by LPS administration.
Materials and methods

Animals
Female, random cycling Balb/c mice (Harlan Spaque Dawley, Horst, The Netherlands) were used at 6-12 wk of age. The mice were kept in 12-h light/dark cycles, in a temperature-controlled room (22°C) and received food and water ad libitum. One week before the experiments the mice were housed in groups according to the experimental set-up. The study was approved by the local animal welfare committee. We performed two experiments.

Experiment 1: Acute illness was induced by an intraperitoneal injection of 150 µg LPS (Endotoxin; Lipopolysaccharide (LPS), E.coli 127:B8, Sigma Chemical Co., St. Louis, MO) diluted in 0.5 ml sterile 0.9% NaCl. Control mice received 0.5 ml sterile 0.9% NaCl. At different timepoints after LPS injection (t=0, 4, 8 and 24 hours) 4-5 mice were anaesthetised with isoflurane and euthanised. The liver, pituitary and hypothalamus were obtained.

Experiment 2: In this experiment, LPS was administered as described above and mice were killed at t=0, 1, 2, 3, 4 and 6 hours (n=5). The liver, thyroid (2 in each group), pituitary and hypothalamus were obtained. In both experiments, blood was taken by cardiac puncture and serum was stored at -20°C until analysed. All tissues were stored immediately in liquid nitrogen.

Thyroid hormones
Serum T3 and T4 were measured with in-house RIAs (Wiersinga & Chopra 1982). To prevent inter-assay variation, all samples of one experiment were measured within the same assay.

RNA isolation and Real Time-PCR
mRNA was isolated from the hypothalamus, pituitary, thyroid and 10 mg liver tissue of mice using the Magna Pure apparatus and the Magna Pure LC mRNA isolation kit II (tissue) (Roche Biochemicals, Mannheim, Germany) according to the manufacturer’s protocol and cDNA synthesis was performed with the 1st Strand cDNA synthesis kit for RT-PCR (AMV) (Roche Molecular Biochemicals, Mannheim, Germany). Published primer pairs were used to amplify HPRT (hypoxanthine phosphoribosyl transferase, a housekeeping gene) (Sweet et al. 2001) and IL-1β (Bouaboula et al. 1992). We designed primer pairs for D1, D2, D3, TRβ1, TRβ2, TSHβ, and preproTRH (D1 forward: CATCTCATCCCTCTACCA and reverse: GCATCTTCCCCGACATTT, D2 forward: GATGCTCCCAATTCCAGTGT and reverse: AGTGAAAGGTGGGTAGGTGG, D3 forward: CTACGTCATC
CAGAGTGCA and reverse: CTGTTCATCATAACGGCTCCA, TRβ1 forward: CACCTGGATCCTGACGATGT and reverse: ACAGGTGATGCAGCGATAGT, TRβ2 forward: GTGAATCAGCCTTATACCTG and reverse: ACAGGTGATGCAGCGATAGT, TSHβ forward: TCAACACCACCATCTG TGCT and reverse: TTGCCACACTTGACGCTCCA, preproTRH forward: TCGTGCTAACTGGTATCCCC and reverse: CCCAAATCTCCCTCTCTCTCC). Real Time PCR was performed for the quantitative estimation of the above mentioned mRNAs. Standards for the different mRNAs were prepared from RNA of murine liver or lung. For each mRNA assayed, a standard curve was generated using ten fold serial dilutions of this target standard PCR product and the same primers used to amplify the cDNA. For each gene the standard protocol was optimised by varying MgCl2 concentrations. PCR reactions were set up with cDNA, MgCl2 (25 mM), SybrGreenI (Roche Molecular Biochemicals, Mannheim, Germany), forward and

![Graphs showing relative expression of IL-1β mRNA in hypothalamus, pituitary, thyroid and liver of mice after administration of LPS (●-●) or saline (○-○). Mean values ± SEM are depicted; p-values indicate differences between groups by ANOVA. Statistical difference between groups at a single timepoint is indicated by symbols: *, p<0.05 and **, p<0.01.](image)

**Figure 1.** Relative expression of IL-1β mRNA in hypothalamus, pituitary, thyroid and liver of mice after administration of LPS (●-●) or saline (○-○). Mean values ± SEM are depicted; p-values indicate differences between groups by ANOVA. Statistical difference between groups at a single timepoint is indicated by symbols: *, p<0.05 and **, p<0.01.
reverse primer and H2O. The reactions were then cycled in the LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) with the following parameters: pre-denaturation for one cycle at 95°C for 10-30 s, amplification for 35-45 cycles (temperature transition of 20 °C/s), which consists of denaturation 0-5 s at 95 °C, annealing at various temperatures for 10 s and elongation for 15 s at 72 °C (annealing temperature D1: 52°C, D2: 55°C, D3: 62°C, TRβ1: 54°C, TRβ2: 55°C, TSHβ: 55°C, preproTRH: 55°C, IL-1β: 60°C, and HPRT: 54°C). Fluorescence reading was taken at 72 °C, melting curve analysis with continuous fluorescence reading. The LightCycler software generated a standard curve (measurements taken during the exponential phase of the amplification) which enabled the amount of each gene in each test sample to be determined. All results were corrected as to their mRNA content using HPRT mRNA.

Liver 5'-deiodinase activity

Liver D1 activity was determined as described before (Peeters et al. 2003). Briefly, mouse liver samples were homogenised on ice in 10 volumes of PE buffer [0.1M phosphate and 2 mM EDTA (pH 7.2)] using a Polytron (Kinematica AG, Lucerne, Switzerland). Homogenates were snap frozen in aliquots and stored at -80°C until further analysis. Protein concentration was measured with the Bio-Rad protein assay using BSA as the standard following the manufacturer’s instructions. D1 activity was measured by duplicate incubations of homogenates (10 μg protein) for 30 min at 37°C with 0.1 μM [3',5'-125I] rT3 (100,000 cpm) in a final volume of 0.1 ml PED10 buffer (PE + 10 mM DTT). Reactions were stopped by addition of 0.1 ml 5% (wt/vol) BSA in water on ice. The protein-bound iodothyronines were precipitated by addition of 0.5 ml ice-cold 10% (wt/vol) trichloroacetic acid in water. Following

![Figure 2. Relative expression of hypothalamic D2 mRNA in mice after administration of LPS (•••) or saline (○○) within 24 hours (big graph) and shortly after LPS administration (inlay). Mean values ± SEM are depicted; p-values indicate differences between groups by ANOVA. Statistical difference between groups at a single timepoint is indicated by symbols: *, p<0.05 and **, p<0.01](image)
centrifugation, $^{125}$I was isolated from the supernatant by chromatography on Sephadex LH-20 minicolumns.

**Figure 3.** Relative expression of TSHβ, TRβ1, TRβ2, D1 and D2 mRNA in the pituitary of mice after administration of LPS (•••) or saline (○○). Mean values ± SEM are depicted; p-values indicate differences between groups by ANOVA. Statistical difference between groups at a single timepoint is indicated by symbols: *, p<0.05 and **, p<0.01
Statistics

Data are presented as the mean ± SEM. Variation between LPS-treated and saline-treated mice were evaluated by analysis of variance (two-way ANOVA) with two grouping factors (time and treatment). Post-hoc analysis (Tukey test) was performed in order to make multiple comparisons between the groups (Hochberg & Tamhane 1987). If the data were abnormal distributed or variances between groups were unequal, we first ranked the data, then performed an ANOVA (Hora 1984) and used a Tukey test for the Post Hoc analyses (all analysed in SPSS 11.5.1, (SPSS Inc. Chigaco, IL)). The differences in serum T3 and T4 at 24 hours and in pituitary D2 mRNA expression at 4 hours were analysed by the Student t-test or by the Mann Whitney U test where appropriate. P-values less than 0.05 were considered as statistically significant.

Results

Hypothalamus

LPS administration resulted in strongly elevated IL-1β mRNA expression in the hypothalamus reaching a maximum after 3 hours (fig. 1). Hypothalamic preproTRH mRNA expression did not change compared to control mice 1-24 hours after LPS administration. Hypothalamic TRβ1 mRNA expression was also not influenced by LPS administration. TRβ2 mRNA expression could hardly be detected (data not shown). By contrast, LPS administration induced a significant 3-fold increase in D2 mRNA expression (maximal at 6–8 h) (fig 2). Hypothalamic D3 mRNA expression was low and not different between LPS-treated and control mice (data not shown).
Figure 5. Relative expression of liver TRβ1 and D1 mRNA, liver D1 activity and serum T₃ and T₄ levels in mice after administration of LPS (•••) or saline (o-o). Mean values ± SEM are depicted; p-values indicate differences between groups by ANOVA. Statistical difference between groups at a single timepoint is indicated by symbols: *, p<0.05 and **, p<0.01.
Pituitary
In the pituitary, LPS induced high IL-1β mRNA expression, maximal at 1h after administration (fig. 1). LPS resulted also in a significant decrease in TSHβ, TRβ2 and D1 mRNA expression within 24 hours, with lowest mRNA expression at 4 hours after LPS administration. D2 mRNA expression and TRβ1 mRNA expression tended to decrease after LPS administration compared to control mice but this difference was not statistically different (fig. 3). However, pituitary D2 mRNA expression at 4 hours was significant lower in LPS treated animals compared to controls.

Thyroid
Thyroidal IL-1β mRNA expression was induced shortly after LPS administration (maximal expression at 1h) (fig. 1). LPS resulted in a significant decrease of thyroidal TSH-R mRNA expression; a reduction in D1 mRNA expression did not reach a statistically significant difference (fig. 4). TRβ1 mRNA expression was not different between LPS-treated and control mice (data not shown).

Figure 6. Model of the timecourse of central and peripheral thyroid hormone metabolism during acute illness in mice induced by LPS administration. LPS rapidly induces IL-1β mRNA expression in all organs of the HPT-axis (see fig. 1) which is followed by almost simultaneous involvement of hypothalamus, pituitary, thyroid and liver in the down-regulation of thyroid hormone metabolism during illness.
Liver and circulation

LPS administration resulted in strongly elevated IL-1β mRNA expression in the liver, which was maximal at 1h and remained elevated until 6 hours (fig. 1). LPS decreased liver TRβ1 and D1 mRNA expression within 8 hours after administration. Liver D1 activity was also significantly decreased after LPS administration, which is in agreement with mRNA expression. The decrease of TRβ1 mRNA preceded the decrease of D1 mRNA expression, which was in turn followed by decreased serum T₃ and T₄ levels after 24 hours (see fig 5).

Discussion

The aim of the present study was to evaluate the time course of changes in central and peripheral thyroid hormone metabolism during acute illness. To this end we injected mice with LPS, which is a very potent activator of the immune system and induces several proinflammatory cytokines via the Toll-like receptor-4 (Lohrer et al. 2000). These cytokines are produced by a variety of cells in many organs. We have measured IL-1β mRNA expression in the HPT-axis as a representative of the inflammatory response and observed a strong induction in hypothalamus, pituitary, thyroid and liver. IL-1β mRNA expression peaked very rapidly (within 1h) in the pituitary, thyroid and liver while hypothalamic IL-1β mRNA expression was maximal at 3-4 hours after LPS administration.

Hypothalamic D2 mRNA expression increased following the rise in IL-1β mRNA, reaching peak values at 6-8 hours after LPS. This did however not result in a change of preproTRH mRNA which – by the presumed rise in local T₃ generation via enhanced D2 activity – indicates altered thyroid hormone feedback. The reason why we could not detect TRβ2 mRNA in the hypothalamus can be that TRβ2 is present only in specific highly localized areas of the hypothalamus (Cook et al. 1992; Lechan et al. 1994). Since in the present experiment we isolated RNA from the complete hypothalamus, it is very possible that this lead to a dilution of the specific signal.

The time course of events in the various organs suggests that LPS induces changes almost simultaneously in the central (hypothalamus and pituitary) and peripheral (thyroid and liver) part of the HPT-axis. In particular, the early changes in the pituitary gland cannot be explained from prior changes in the hypothalamus. One may argue that the changes in the thyroid gland may be to some extent secondary to a fall in pituitary TSH release. This however seems unlikely since downregulation of the TSH-R has been described as a result of higher ambient TSH concentrations (Shimura et al. 1997) whereas we observed down regulation in thyroidal TSH-R mRNA expression associated with low TSHβ mRNA expression. Our results
are consequently best interpreted as independent effects of LPS on the various components of the HPT-axis during the early stages of induced illness. In more advanced stages down-regulation at the hypothalamic level may enhance down-regulation at the pituitary level, which then may further contribute to decreased secretion of thyroid hormones by the thyroid.

Some of the alterations in the HPT-axis we observed have been described previously. The decrease of pituitary D1 and D2 mRNA expression (only after 4 hrs) confirms our recent observations (Boelen et al. 2004). The decrease in liver D1 mRNA expression, which precedes the decrease in serum thyroid hormones, has also been described previously by us (Boelen et al. 1995). Unaltered hypothalamic preproTRH mRNA expression in rats after LPS administration is reported by Kakucska et al. (Kakucska et al. 1994), and the increase in hypothalamic D2 expression we find is consistent with a recent observation of an increase in D2 activity in the mediobasal hypothalamus (MBH) of the rat during acute LPS-induced illness (Fekete et al. 2003). Recently, Diano et al. showed that three days fasting also induced an increase in D2 mRNA expression in the hypothalamus of rats possible resulting from decreased plasma leptin or elevated glucocorticoids levels (Diano et al. 1998). The effect of food deprivation as a result of illness can be excluded in our study because D2 mRNA expression starts to rise shortly after LPS administration. At that moment, the effect of food deprivation on thyroid hormone metabolism is negligible (Boelen et al. 1995).

Data on changes in the human hypothalamus during critical illness are sparse. Arem et al. reported a decreased T3/T4 ratio in post-mortem human hypothalamus of patients who died after protracted illness as compared with patients who died acutely, suggesting decreased deiodination of T4 in human illness (Arem et al. 1993). However, D2 activity was not assessed in their study. Furthermore, decreased deiodination is hard to reconcile with decreased TRH mRNA expression in the PVN of patients with critical illness as reported in our earlier studies (Fliers et al. 1997), or with true unaltered hypothalamic TRH expression in the present study. At least two factors should be taken into account interpreting these findings. First, LPS-induced increased D2 expression in the mediobasal hypothalamus as reported by Fekete et al in the rat (Fekete et al. 2003) and replicated by the present study in the mouse probably represents a more acute disease model than the patients studied by Arem et al. and Fliers et al. (Arem et al. 1993; Fliers et al. 1997). Second, only a small proportion of hypothalamic TRH expressing neurons is involved in HPT axis feedback regulation (Segerson et al. 1987). This may therefore obscure effects of LPS that are restricted to selected hypothalamic nuclei such as the PVN when studying hypothalamic tissue blocks, probably explaining unaltered hypothalamic TRH mRNA expression as found in the present study.

Our results suggest simultaneous involvement of the hypothalamus, pituitary, thyroid and liver in the down-regulation of thyroid hormone metabolism during
Changes in the HPT-axis during acute illness. It is logical to assume a general mechanism inducing these changes in various organs since the changes are viewed as part of the acute phase response during illness. Cytokines have been implicated in the pathogenesis of altered thyroid hormone metabolism during illness (Boelen et al. 1996). It has been hypothesized recently that limiting amounts of SRC-1, a coactivator for both NF-κB and TRβ1, might be responsible for decreased liver D1 mRNA expression because of competition between cytokine-induced NF-κB and the TRβ1/D1 complex for SRC-1 (Yu & Koenig 2000). A similar mechanism could also play a role in the pituitary, because the simultaneous decrease of pituitary TRβ2 and TSHβ mRNAs we observe suggests a shortage of a common factor needed for the expression of both TRβ2 and TSHβ. One candidate could be the pituitary specific transcription factor Pit-1, which is necessary for the expression of both genes (Shupnik 2000).

The early decrease in liver TRβ1 mRNA expression, which occurs prior to and probably contributes to the decrease of liver D1 mRNA might be caused by other mechanisms, for instance the recently described participation of the ubiquitine proteasome in TR degradation (Englebienne et al. 2003).

Our results, presented schematically in figure 6, suggest involvement of the hypothalamus, pituitary, thyroid and peripheral tissues (liver) in the down-regulation of thyroid hormone metabolism during acute illness. Although the mechanisms remain to be established, competition for limiting amounts of nuclear factors involved in both immune response and thyroid hormone metabolism seems an attractive possibility to explain at least partly of the central and peripheral changes in this condition.

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Chapter 2

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