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

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Differential involvement of NFκB and AP-1 pathways in the IL-1β-mediated decrease of Deiodinase type 1 and Thyroid Hormone Receptor β1 mRNA

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Abstract

One of the hallmarks of the sick euthyroid syndrome or the nonthyroidal illness syndrome is a decrease of serum T₃, caused mainly by a decrease in liver D1 mRNA and activity. Proinflammatory cytokines like IL-1β are likely involved in this disease, but are also known to inhibit TRβ1 gene expression, which is of interest as the D1 promoter contains TRE's. The aim of the present study was to evaluate whether the IL-1β induced decrease of D1 and TRβ1 mRNA is mediated by the same cytokine signalling pathways in a human hepatoma cell-line (HepG2). We observed a down regulation of both D1 and TRβ1 mRNA after 4 hours of incubating the cells with IL-1β. Sulfasalazine was used to inhibit the NFκB pathway and SP600125, a chemical inhibitor of the c-Jun N-terminal kinase, was used as an inhibitor of the AP-1 pathway. AP-1 inhibition did not affect the decrease of D1 and TRβ1 mRNA, but TRβ1 mRNA decrease was completely abolished after inhibiting NFκB while D1 mRNA was unaffected. Only simultaneous inhibition of both the NFκB and AP-1 pathway abolished D1 mRNA decrease. We conclude that IL-1β stimulation of HepG2 cells results in a marked decrease of D1 and TRβ1 mRNA. The decrease of TRβ1 mRNA is exclusively mediated by the NFκB pathway, while the decrease of D1 mRNA requires inhibition of both the AP-1 and the NFκB pathway.
Introduction

The nonthyroidal illness syndrome (NTIS) (also known as the euthyroid sick syndrome) is characterized by a decrease in serum T₃ without an increase in serum TSH. Proinflammatory cytokines are involved in the characteristic decrease of liver 5'-deiodinase type 1 (D1) mRNA expression and activity during illness, which contributes to the diminished serum T₃ levels. (Boelen et al. 1995; Boelen et al. 1996; Wiersinga 2000; Jakobs et al. 2002). D1 mRNA expression in liver is positively regulated by T₃, primarily by binding of the liganded Thyroid Hormone Receptor (TR)β1 to TRE’s in the promoter region of the D1 gene (Amma et al. 2001) (Jakobs et al. 1997). The induction of proinflammatory cytokines by bacterial endotoxin (Lipopolysaccharide (LPS)) in mice not only results in a decreased D1 mRNA expression and activity in liver, but also in a decrease of TRβ1 mRNA and protein in liver (Boelen et al. 2004; Beigneux et al. 2003). The pathways mediating these changes are incompletely understood.

In vitro studies have shown that IL-1β, IFNγ and IL-6 decrease D1 promoter activity in the Hepatocellular Carcinoma cell-line (HepG2), IL-1β being the most potent (Jakobs et al. 2002). Nagaya et al showed that addition of the antibiotic Clarithromycin inhibits the IL-1β induced decrease of D1 mRNA in HepG2 cells and claimed the involvement of the NFkB pathway (Nagaya et al. 2000). However, Clarithromycin turns out to be an inhibitor of both the NFkB and AP-1 signaling pathways (Kikuchi et al. 2002) and the influence of the AP-1 pathway on the D1 mRNA decrease was not evaluated. The transcription factors NFkB and AP-1 are important mediators of cytokine production, but it is unclear how these signaling pathways interact in mediating the D1 and TRβ1 mRNA decrease.

The NFkB pathway is activated by LPS or cytokines through phosphorylation of IkB Kinases (IKK) which in turn phosphorylate the inhibitory proteins IkB’s that are bound to the NFkB transcription factors in the cytoplasm. Phosphorylated IkB’s are degraded by the 26S proteasome, leaving the NFkB transcription factors free to activate gene-transcription in the nucleus. A specific NFkB inhibitor is Sulfasalazine. It inhibits IKK and therefore inhibits the activation of the NFkB pathway (Weber et al. 2000).

SP600125 is a compound which specifically inhibits c-Jun N-terminal kinase (JNK), which is involved in the AP-1 pathway. In response to LPS or cytokine stimulation JNK is activated by phosphorylation and phosphorylates the AP-1 transcription factors fos and jun, which upon phosphorylation activate gene-transcription. SP600125 is known to inhibit LPS induced TNFα production in vivo and to decrease cytokine mRNA expression in human monocytes after LPS stimulation in vitro (Bennett et al. 2001).

The aim of our study was to evaluate the role of NFkB and AP-1 activation in the cytokine induced decrease of D1 and TRβ1 mRNA. We used IL-1β stimulation
of HepG2 cells in order to study the cytokine induced decrease of D1 in vitro. We first show that IL-1β stimulation of HepG2 cells not only results in D1 mRNA decrease, but also induces a decrease of TRβ1 mRNA. By using the specific AP-1 inhibitor SP600125 and the specific NFκB inhibitor Sulfasalazine separately and simultaneously, we specify the influence of NFκB en AP-1.

Materials and Methods

Cell cultures
The human hepatoma cell line, HepG2 (ATCC, Rockville, USA) was cultured in EMEM, supplemented with 10 U/ml of penicillin, streptomycin, fungizone and 5% Fetal Calf Serum (all from Cambrex, East Rutherford, USA). For the RNA-expression studies, 5*10^4 cells per well were grown for 24 hours in a 24-wells plate. For protein expression studies 3*10^6 cells were grown in a 75cm² culture-flask for 4 days. Cells were stimulated with 10 ng/ml IL-1β (Sigma, St. Louis, USA) dissolved in phosphate-buffered saline with 0.5% (w/v) BSA (PBS/BSA). PBS/BSA was added in the same amount in the negative control. For the inhibition of AP-1, SP600125 (Calbiochem, Darmstadt, Germany) dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, USA) was used in concentrations of 10 µM, 20 µM and 50 µM. DMSO was added in control stimulations reaching concentrations of 0.1%, 0.2% and 0.5% (v/v). Sulfasalazine (Sigma, St. Louis, USA) was used as a NFκB inhibitor. Sulfasalazine was dissolved in DMSO and added in the concentrations 2 and 3 mM. As a control DMSO was added in concentrations of 0.8% and 1.2% (v/v). Cells were pre-incubated for 30 min with SP600125, Sulfasalazine or with both SP600125 and Sulfasalazine. At re-incubation, IL-1β was added without washing the cells. Experiments for mRNA analysis were done in duplicate; a representative experiment is shown in the figures.

RNA isolation and RT-PCR
For RNA isolation cells were washed with PBS and subsequently lysed in 200 µl lysis buffer from the Magna Pure LC RNA Isolation kit-High Performance (Roche Molecular Biochemicals, Mannheim, Germany). The RNA was isolated on the Magna Pure (Roche Molecular Biochemicals, Mannheim, Germany) using the protocol and buffers supplied with the Magna Pure LC RNA Isolation kit-High Performance. Total RNA amounts were measured using the Nanodrop (Nanodrop, Wilmington, Delaware USA). cDNAsynthesis was performed using the First Strand cDNA Synthesis Kit for RT-PCR with oligo d(T) primers and equal RNA-input (Roche Molecular Biochemicals, Mannheim, Germany). Real Time PCR was performed using the
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Lightcycler (Roche Molecular Biochemicals, Mannheim, Germany). For all PCR’s Lightcycler DNA Master SYBR Green I kit (Roche Molecular Biochemicals, Mannheim, Germany) was used, adding 3mM MgCl2 and 50ng primers (Biologio, Nijmegen, The Netherlands) each. We designed primer pairs for D1 (forward: 5’-AGC CAC GAC AAC TGG ATA CC-3’, reverse: 5’-ACT CCC AAA TGT TGC ACC TC-3’), primers for HPRT and TRβ1 were previously described (Liu et al. 2003; Silva et al. 2002). PCR programs were as follows: denaturation 30 sec 95°C, 40-45 cycles of 0-5 sec 95°C, 10 sec annealing temperature, 15-20 sec 72°C. Annealing temperatures were: 60°C for HPRT, 57°C for D1 and 56°C for TRβ1. For quantification a standard curve was generated of a sequence-specific PCR-product ranging from 0.01 fg/μl until 100 fg/μl. Samples were corrected for their RNA content using HPRT as a housekeeping gene. Samples were individually checked for their PCR-efficiency (Ramakers et al. 2003). The median of the efficiency was calculated for each assay, samples that had a greater difference than 0.05 of the efficiency median value, were not taken into account.

**Protein isolation and Western blotting**

Protein was isolated by washing and scraping the cells in ice-cold PBS. After centrifugation for 5 min at 500g, the cell pellet was lysed in 250 μl of 2x SDS sample buffer (2% (w/v) SDS, 10% (v/v) glycerol, 100mM DTT, 60mM Tris pH 6.8, broomphenolblue). The samples were boiled for 5 min. 5 μl of each sample was loaded on a 10% SDS/PAGE gel (Bio-Rad, Hercules, USA). For quantification of D1 and TRβ1 protein in whole cell extract (WCE) the pellet was dissolved in Sol A (20 mM Tricine pH 7.6, 2 mM CaCl2, 1 mM MgCl2, 5%(v/v) glycerol, 0.25 M Sucrose and protease inhibitor cocktail (Roche, Mannheim, Germany)). For TRβ1 protein nuclei and WCE were isolated using a glass-teflon homogenizer. Nuclei were dissolved in SolB (20 mM Tris pH 7.6, 1 mM EDTA, 50 mM NaCl, 5%(v/v) glycerol, 0.25 M Sucrose and protease inhibitor cocktail (Roche, Mannheim, Germany)). Protein content was measured and 10 μg (WCE) or 5 μg (nuclear extract) was loaded on a 10% SDS-PAGE gel. Gels were blotted on Immobilon-P Transfer Membrane membrane (Millipore, Bedford, USA). Blots were blocked with 2% (w/v) non-fat milk in Tris-buffered saline (TBS)-Tween (TRβ1 in 1% (w/v) casein in TBS-Tween, D1 in 3% (w/v) casein in PBS-0.01%Tween) for 1 hour at RT. Primary antibody’s used for protein detection were: anti-β-Actin (I-19) (Santa Cruz Biotechnology Inc., Santa Cruz, USA), anti-phospho-JNK (9251) and anti-phospho-IκBα (9246) (Cell Signalling Technology, Beverly, USA), anti-D1 (Leonard et al. 2001) and anti-TRβ1(#319) (Zandieh et al. 2002). All antibody’s were 1:1000 diluted, except for anti-β-Actin (1:2000) anti-D1 (1:50) and anti-TRβ1 (1:500). Incubation with the primary antibody was performed for 1 hour at RT, then overnight at 4°C. Blots were washed 4 times 2 min with TBS-Tween (PBS-Tween
for D1). As secondary antibody goat-anti-mouse-Horse-Radish-Peroxidase (HRP) (phospho-I\textsuperscript{kB}a), goat-anti-rabbit-HRP (phospho-JNK, TR\textbeta{}1, D1) or rabbit-anti-goat-HRP (β-actin) (DAKO Cytomation, Glostrup, Denmark) was used, 1:2000 diluted (1:20,000 for β-actin, D1 and TR\textbeta{}1), incubation for 1 hour at RT. After incubation the blots were washed with TBS-Tween (PBS-Tween for D1) 4 times 2 min. Subsequently Lumi-Light\textsuperscript{Plus} chemiluminescent substrate was added (Roche Molecular Biochemicals, Mannheim, Germany). The emitted light was visualized and quantified on the Lumi-Imager (Roche Molecular Biochemicals, Mannheim, Germany). Samples were corrected for their protein content using β-actin or by loading equal amounts of protein.

Statistics
Non-parametric Mann-Whitney U tests (SPSS, Chicago, USA) were performed to test statistical significance between groups. For time-course experiments statistical significance was tested by two-way ANOVA (Excel Microsoft, Redmond, USA).

Results

D1 and TR\textbeta{}1 mRNA decrease after IL-1β stimulation
A time-course experiment was performed to study the IL-1β induced decrease of D1 and TR\textbeta{}1 mRNA. HepG2 cells were incubated with 10 ng/ml IL-1β. After 0, 2, 4 and 6 hours of IL-1β stimulation RNA was isolated and D1 and TR\textbeta{}1 mRNA were measured. Both D1 and TR\textbeta{}1 mRNA significantly decreased after IL-1β stimulation ($P<0.01$). This difference was significant after 4 and 6 hours as compared to controls (Fig. 1A). No difference in time-course of the IL-1β induced decrease of D1 and TR\textbeta{}1 mRNA was observed. The IL-1β induced decrease of D1 mRNA resulted in decreased D1 protein levels at 24 hours after IL-1β. TR\textbeta{}1 protein levels however did not change after IL-1β, neither in the WCE after 4, 8, 12, 16, 24 or 48h of IL-1β nor in the nuclear extract after 16, 24 and 48h of IL-1β (Fig 1B).

The AP-1 pathway has no influence on the decrease of D1 and TR\textbeta{}1 mRNA
To investigate the influence of the AP-1 signalling pathway on the IL-1β induced decrease of D1 and TR\textbeta{}1 mRNA, SP600125, a specific AP-1 inhibitor was used. HepG2 cells were 30 minutes pre-incubated with 10, 20 and 50 μM SP600125 before IL-1β was added. Microscopically no changes in cell morphology were seen after incubation with SP600125. Protein analysis showed that 50 μM SP600125 indeed inhibited the phosphorylation of JNK and c-jun, which are markers for AP-1 activation (Fig. 2A). After 4 hours of IL-1β stimulation RNA was isolated and D1 and TR\textbeta{}1 mRNA was
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Figure 1. A) D1 and TRβ1 mRNA expression in HepG2 cells after 0, 2, 4 and 6 hours in controls (○) and after IL-1β (●). Mean values ± SEM (n=6) are shown. P values indicate differences between groups by ANOVA. Significances of separate time-points were evaluated by Mann-Whitney U tests, ** P≤0.01, B) D1 and TRβ1 protein expression in WCE of HepG2 cells after 24 hours after IL-1β compared to controls.

The NFκB pathway only influences the IL-1β induced TRβ1 mRNA decrease

The IKK inhibitor Sulfasalazine was used to inhibit the NFκB pathway in order to see whether the IL-1β mediated decrease of D1 and TRβ1 mRNA was influenced by NFκB. 30 minutes before IL-1β stimulation, HepG2 cells were incubated with 2 and 3 mM Sulfasalazine. Protein analysis showed that after pre-incubation with 3 mM Sulfasalazine, IL-1β stimulation did not result in phosphorylation of IκBα in the Sulfasalazine treated cells compared to the vehicle DMSO control (Fig. 3A), which shows that the NFκB pathway is indeed inhibited. After 4 hours of IL-1β stimulation RNA was isolated. mRNA analysis showed that the TRβ1 mRNA decrease was abolished by 2 and 3 mM Sulfasalazine compared to the vehicle DMSO control (P=0.047 and P=0.009). The decrease of D1 mRNA was unaffected by Sulfasalazine treatment (Fig 3B). Because of the toxic effects of Sulfasalazine, which could be seen microscopically in changed cell morphology, we evaluated whether Sulfasalazine alone affected the TRβ1 and D1 mRNA expression. However, adding 3 mM Sulfasalazine did not significantly change the mRNA expression of TRβ1 and D1 (data not shown).
Simultaneous inhibition of the AP-1 and NFκB pathways abolishes the decrease of both TRβ1 and D1 mRNA

To inhibit the AP-1 and NFκB pathways simultaneously, HepG2 cells were 30 minutes pre-incubated with 50 µM SP600125 and 3 mM Sulfasalazine. Cells were subsequently stimulated with IL-1β for 4 hours. RNA was isolated and D1 and TRβ1 mRNA was measured. The IL-1β induced decrease of TRβ1 and D1 mRNA was abolished by inhibition of both the NFκB and AP-1 pathway, as is shown in figure 4A and 4B (P=0.004 for D1 and TRβ1).
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Figure 3. A) Protein expression in HepG2 cells after 0, 15, 30, 60 and 120 minutes IL-1β stimulation, after 30 minutes pre-incubation with either 3 mM Sulfasalazine + DMSO (right panel) or vehicle DMSO (left panel). Western blots were detected with anti-phospho-IκBα (upper panel) and anti-β-actin (lower panel) antibodies. B) Relative expression of D1 and TRβ1 mRNA in HepG2 cells. Medium control (black bar), 4 hours IL-1β stimulation (white bar), 4 hours IL-1β stimulation with 30 minutes pre-incubation with 2 or 3 mM Sulfasalazine (dotted bars) or vehicle DMSO (grey bars). Mean values ± SEM (n=6) are shown. Significances were evaluated by Mann-Whitney U tests, * P≤0.05, ** P≤0.01.

Figure 4. Relative expression of D1 and TRβ1 mRNA in HepG2 cells. Medium control (black bar), 4 hours IL-1β stimulation (white bar), 4 hours IL-1β stimulation with 30 minutes pre-incubation of 3 mM Sulfasalazine and 50 µM SP600125 (dotted bar) or vehicle DMSO (grey bar). Mean values ± SEM (n=6) are shown. Significances were evaluated by Mann-Whitney U tests, ** P≤0.01.
Figure 5. A) Schematic overview of the possible mechanisms behind the IL-1β-mediated decrease of TRβ1 mRNA expression: Repressed gene transcription based on the presence of three possible NFκB binding-sites in the TRβ1 promoter, and NFκB mediated increase of TRβ1 mRNA degradation. B) Schematic overview of the possible mechanisms behind the IL-1β-mediated decrease of D1 mRNA expression: Competition for limiting amounts of coactivators of gene-transcription.


Discussion

The aim of our study was to evaluate the role of NFκB and AP-1 activation in the IL-1β-induced decrease of liver D1 and TRβ1 mRNA, which occurs in liver of mice after LPS administration, an animal model of (acute) NTIS. IL-1β stimulation of HepG2 cells resulted in a D1 and TRβ1 mRNA decrease, which is in agreement with our in vivo results (Boelen et al. 2004). The D1 mRNA decrease resulted in a decrease of D1 protein levels after 24h of IL-1β stimulation, which is in agreement with the study of Jakobs et al. The TRβ1 mRNA decrease however did not result in a decrease of TRβ1 protein levels in contrast to the in vivo studies by Beigneux et al. This could be explained by differences between the in vitro and in vivo experimental setting: adding IL-1β to the cell culture once might be completely different from the complex cascade of reactions caused by LPS administration in mice. Probably other posttranslational events, induced by LPS or other cytokines are necessary to decrease the amount of TRβ1 protein. We specified the influence of NFκB en AP-1 activation by using the specific AP-1 inhibitor SP600125 and the specific NFκB inhibitor Sulfasalazine separately and simultaneously. Sulfasalazine prevented IkBα phosphorylation and SP600125 prevented JNK phosphorylation, demonstrating that the inhibitors worked effectively.

Inhibition of the AP-1 pathway by SP600125 did not change the TRβ1 mRNA decrease, but inhibition of NFκB by Sulfasalazine prevented the IL-1β–induced TRβ1 mRNA decrease. This effect could not be due to the toxic effect of Sulfasalazine on the cells, because incubation with Sulfasalazine alone did not change the TRβ1 mRNA expression. It is not clear whether the TRβ1 mRNA decrease is due to an NFκB-mediated decrease in transcriptional activation of the TRβ1 promoter or an NFκB-mediated specific increase in TRβ1 mRNA degradation. A transcription element search system (TESS) analysis showed that there are 3 possible NFκB binding sites present in the TRβ1 promoter (Schug & and Overton GC 1998). It is possible that these sites are responsible for NFκB-mediated transcriptional repression of the TRβ1 gene. mRNA degradation during inflammation has been reported of Connexin32, Retinoid X Receptor(RXR)β and RXRγ (Theodorakis & De Maio 1999) (Beigneux et al. 2000). Increased Connexin 32 mRNA degradation during inflammation was due to increased deadenylation of the poly(A) tail whereas the increased RXRβ and RXRγ mRNA degradation was proven by observing a decrease in mRNA expression without a change in promoter activity. A combination of altered promoter activity and increased RNA degradation is also a possibility. Beigneux et al have shown that a marked decrease of RXRα mRNA during inflammation could not be the result of the mild decrease of RXRα promoter activity, indicating a combination of mechanisms (Beigneux et al. 2000). A schematic overview of these mechanisms is shown in figure 5A. It can be hypothesized that reduced levels of TRβ1 mRNA and subsequently reduced

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levels of TRβ1 protein are responsible for the decrease of TRβ1-mediated D1 gene transcription. However, because of the rapid decrease of both TRβ1 and D1 mRNA at the same time it seems unlikely that reduced TRβ1 protein levels are responsible for the early D1 mRNA decrease. This is underlined by our finding that TRβ1 protein levels of HepG2 cells after IL-1β stimulation are not reduced compared to controls. D1 mRNA decreases just as fast as TRβ1 after IL-1β stimulation of HepG2 cells. However, our experiments showed that the mechanism behind the D1 mRNA decrease is different from the TRβ1 mRNA decrease. Inhibition of the NFκB pathway or the AP-1 pathway did not prevent the IL-1β–induced decrease of D1 mRNA. Simultaneous inhibition of NFκB and AP-1 however prevented the effect of IL-1β on D1 mRNA expression, which is in agreement with the Clarithromycin experiments of Nagaya et al. Both the NFκB and AP-1 pathways use common factors, like coactivators, in order to activate transcription of cytokine-regulated genes. Some of these factors also play a role in TRβ1-mediated D1 gene transcription, like the coactivators Steroid Receptor Coactivator (SRC)-1 and CREB-binding protein (CBP). Yu et al showed that the IL-1β-induced decrease of D1 gene transcription in rat hepatocytes can be partially overcome by adding exogenous SRC-1. The authors therefore hypothesized that limiting amounts of coactivators could play a role in the cytokine-induced decrease of D1 mRNA (Yu & Koenig 2000). This hypothesis is supported by the study of Jakobs et al, who showed that the cytokine-induced decrease of D1 mRNA in HepG2 cells was due to a decrease of transcriptional activation of the D1 promoter, which could be caused by a shortage of coactivators (Jakobs et al. 2002). Our results are in line with these studies because the IL-1β–induced decrease of D1 was not related to one specific inflammatory pathway and thus probably caused by a shortage of common factors used by both inflammatory pathways and TRβ1-mediated D1 gene transcription, like SRC-1. A schematic overview of this mechanism is shown in figure 5B.

In summary: The IL-1β-induced D1 and TRβ1 mRNA decrease in HepG2 cells are regulated by different mechanisms. The IL-1β–induced decrease of TRβ1 mRNA is regulated by NFκB, but the exact mechanism remains unknown. Possibilities are an NFκB related increase of TRβ1 mRNA degradation or a decreased transcriptional activation of the TRβ1 gene. A possible mechanism involved in the D1 mRNA decrease might be competition for limiting amounts of common factors involved in transcriptional activation of NFκB, AP-1 and TRβ1-mediated D1 gene-transcription.
References


Ref Type: Report


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