Differential involvement of nuclear factor- κ B and activator protein-1 pathways in the interleukin-1 β -mediated decrease of deiodinase type 1 and thyroid hormone receptor β 1 mRNA

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Abstract

One of the hallmarks of the sick euthyroid syndrome or non-thyroidal illness is a decrease of serum triiodothyronine, caused mainly by a decrease in liver deiodinase type 1 (D1) mRNA and activity. Proinflammatory cytokines like interleukin (IL)-1 β are likely involved in this disease, but are also known to inhibit thyroid hormone receptor (TR)- β 1 gene expression, which is of interest as the D1 promoter contains TREs. The aim of the present study was to evaluate whether the IL-1 β -induced decrease of D1 and TR β 1 mRNA is mediated by the same cytokine signalling pathways in a human hepatoma cell line (HepG2). We observed a downregulation of both D1 and TR β 1 mRNA after 4 h of incubating the cells with IL-1 β . Sulfasalazine was used to inhibit the nuclear factor- κ B (NF κ B) pathway

and SP600125, a chemical inhibitor of the c-Jun N-terminal kinase, was used as an inhibitor of the activator protein-1 (AP-1) pathway. AP-1 inhibition did not affect the decrease of D1 and TR β 1 mRNA, but the TR β 1 mRNA decrease was completely abolished after inhibiting NF κ B, while D1 mRNA was unaffected. Only simultaneous inhibition of both the NF κ B and AP-1 pathways abolished the D1 mRNA decrease. We concluded that IL-1 β stimulation of HepG2 cells results in a marked decrease of D1 and TR β 1 mRNA. The decrease of TR β 1 mRNA is exclusively mediated by the NF κ B pathway, while the decrease of D1 mRNA requires inhibition of both the AP-1 and the NF κ B pathways.

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Introduction

Non-thyroidal illness (also known as the euthyroid sick syndrome) is characterized by a decrease in serum triiodothyronine (T3) without an increase in serum thyrotrophin. 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, 1996, Wiersinga 2005, Jakobs *et al.* 2002). D1 mRNA expression in liver is positively regulated by T3, primarily by binding of the liganded thyroid hormone receptor (TR)- β 1 to TREs in the promoter region of the D1 gene (Jakobs *et al.* 1997, Amma *et al.* 2001). 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 TR β 1 mRNA and protein in liver (Beigneux *et al.* 2003, Boelen *et al.* 2004). The pathways mediating these changes are incompletely understood.

In vitro studies have shown that interleukin (IL)-1 β , interferon- γ and IL-6 decrease D1 promoter activity in the hepatocellular carcinoma cell line (HepG2), IL-1 β being the most potent (Jakobs *et al.* 2002). Nagaya *et al.* (2000) showed that addition of the antibiotic clarithromycin inhibits the IL-1 β -induced decrease of D1 mRNA in HepG2 cells and claimed the involvement of the nuclear factor- κ B (NF κ B) pathway. However, clarithromycin turns out to be an inhibitor of both the NF κ B and activator protein-1 (AP-1) signalling pathways (Kikuchi *et al.* 2002) and the influence of the AP-1 pathway on the D1 mRNA decrease was not evaluated. The transcription factors NF κ B and AP-1 are important mediators of cytokine production, but it is unclear how these signalling pathways interact in mediating the D1 and TR β 1 mRNA decrease.

The NF κ B pathway is activated by LPS or cytokines through phosphorylation of NF κ B inhibitory protein (I κ B) kinases (IKK) which in turn phosphorylate the inhibitory proteins I κ Bs that are bound to the NF κ B transcription factors in the cytoplasm. Phosphorylated I κ Bs

are degraded by the 26S proteasome, leaving the NFκB transcription factors free to activate gene transcription in the nucleus. A specific NFκB inhibitor is sulfasalazine. It inhibits IKK and therefore inhibits the activation of the NFκB 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 tumor necrosis factor-α 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 NFκB 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 have first shown 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 have specified the influence of NFκB on AP-1.

Materials and Methods

Cell cultures

The human hepatoma cell line HepG2 (ATCC, Rockville, MD, USA) was cultured in Eagle's Minimum Essential Medium (EMEM), supplemented with 10 U/ml penicillin, streptomycin, fungizone and 5% fetal calf serum (all from Cambrex, East Rutherford, NJ, USA). For the RNA expression studies, 5×10^4 cells per well were grown for 24 h in a 24-well plate. For protein expression studies, 3×10^6 cells were grown in a 75 cm² culture flask for 4 days. Cells were stimulated with 10 ng/ml IL-1β (Sigma) dissolved in PBS 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 DMSO (Sigma) 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) was used as an NFκB inhibitor. Sulfasalazine was dissolved in DMSO and added in concentrations of 2 and 3 mM. As a control, DMSO was added in concentrations of 0.8% and 1.2% (v/v). Cells were preincubated 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). The RNA was isolated on the Magna Pure using the protocol and buffers supplied with the Magna Pure LC RNA isolation kit–high performance. Total RNA amounts were measured using Nanodrop (Nanodrop, Wilmington, DE, USA).

CDNA synthesis was performed using the first-strand cDNA synthesis kit for RT-PCR with oligo d(T) primers and equal RNA input (Roche Molecular Biochemicals). Real-time PCR was performed using the Lightcycler (Roche Molecular Biochemicals). For all PCRs, Lightcycler DNA Master SYBR Green I kit (Roche Molecular Biochemicals) was used, adding 3 mM MgCl₂ and 50 ng primers (Biolegio, 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 hypoxanthine phosphoribosyl transferase (HPRT) and TRβ1 were as previously described (Silva *et al.* 2002, Liu *et al.* 2003). PCR programs were as follows: denaturation 30 s at 95 °C, 40–45 cycles of 0–5 s at 95 °C, 10 s at annealing temperature, 15–20 s at 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 up to 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 difference greater 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 500 g, the cell pellet was lysed in 250 μl 2 × SDS sample buffer (2% (w/v) SDS, 10% (v/v) glycerol, 100 mM dithiothreitol, 60 mM Tris, pH 6.8 and bromophenol blue). The samples were boiled for 5 min. Five microlitres of each sample were loaded onto a 10% SDS/PAGE gel (Bio-Rad). For quantification of D1 and TRβ1 protein in whole cell extract (WCE), the pellet was dissolved in solution 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 Molecular Biochemicals)). For TRβ1, protein nuclei and WCEs were isolated using a glass–Teflon homogenizer. Nuclei were dissolved in solution B (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 Molecular Biochemicals)).

Figure 1 (A) D1 and TR β 1 mRNA expression in HepG2 cells after 0, 2, 4 and 6 h in controls (○) and after IL-1 β (●). Mean values $\pm$ S.E.M. ($n=6$) are shown. P values indicate differences between groups by ANOVA. Significances of separate time-points were evaluated by Mann-Whitney U -tests; ** $P \leq 0.01$. (B) D1 and TR β 1 protein expression in WCEs of HepG2 cells 24 h after IL-1 β , compared with controls.

Protein content was measured and 10 μ g (WCE) or 5 μ g (nuclear extract) was loaded on a 10% SDS-PAGE gel. Gels were blotted on Immobilon-P transfer membrane (Millipore, Bedford, MA, USA). Blots were blocked with 2% (w/v) non-fat milk in Tris-buffered saline (TBS)-Tween (TR β 1 in 1% (w/v) casein in TBS-Tween, D1 in 3% (w/v) casein in PBS-0.01% Tween) for 1 h at room temperature (RT). Primary antibodies used for protein detection were: anti- β -actin (I-19) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), anti-phospho-JNK (9251) and anti-phospho-I κ B α (9246) (Cell Signaling Technology, Danvers, MA, USA), anti-D1 (Leonard *et al.* 2001) and anti-TR β 1 (319) (Zandieh *et al.* 2002). All antibodies were 1:1000 diluted, except for anti- β -actin (1:2000) anti-D1 (1:50) and anti-TR β 1 (1:500). Incubation with the primary antibody was performed for 1 h at RT, then overnight at 4 $^{\circ}$ C. Blots were washed four times for 2 min with TBS-Tween (PBS-Tween for D1). As secondary antibodies, goat anti-mouse horseradish peroxidase (HRP) (phospho-I κ B α), goat anti-rabbit HRP (phospho-JNK, TR β 1 and D1) or rabbit anti-goat HRP (β -actin) (DAKO Cytomation, Glostrup, Denmark) were used, 1:2000 diluted (1:20 000 for β -actin, D1 and TR β 1), with incubation for 1 h at RT. After incubation, the blots were washed with TBS-Tween (PBS-Tween for D1) four times for 2 min. Subsequently Lumi-Light^{plus} chemiluminescent substrate was added (Roche Molecular Biochemicals). The emitted light was visualized and quantified on the Lumi-Imager (Roche Molecular Biochemicals). Samples were corrected for their protein content using β -actin or by loading equal amounts of protein.

Statistical analysis

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

Results

D1 and TR β 1 mRNA decrease after IL-1 β stimulation

A time-course experiment was performed to study the IL-1 β -induced decrease of D1 and TR β 1 mRNA. HepG2 cells were incubated with 10 ng/ml IL-1 β . After 0, 2, 4 and 6 h of IL-1 β stimulation RNA was isolated and D1 and TR β 1 mRNA were measured. Both D1 and TR β 1 mRNA significantly decreased after IL-1 β stimulation ($P < 0.01$). This difference was significant after 4 and 6 h as compared with controls (Fig. 1A). No difference in the time-course of the IL-1 β -induced decrease of D1 and TR β 1 mRNA was observed. The IL-1 β -induced decrease of D1 mRNA resulted in decreased D1 protein levels at 24 h after IL-1 β . TR β 1 protein levels, however, did not change after IL-1 β , either in the WCE after 4, 8, 12, 16, 24 or 48 h of IL-1 β or in the nuclear extract after 16, 24 and 48 h of IL-1 β (Fig. 1B).

The AP-1 pathway has no influence on the decrease of D1 and TR β 1 mRNA

To investigate the influence of the AP-1 signalling pathway on the IL-1 β -induced D1 and TR β 1 mRNA decrease,

Figure 2 (A) Protein expression in HepG2 cells after 0, 15, 30, 45 and 60 min of IL-1β stimulation, following preincubation for 30 min with either 50 μM SP600125+DMSO (right panel) or vehicle DMSO (left panel). Western blots were detected with anti-phospho-JNK (upper panel), anti-phospho-c-jun (middle panel) and anti-β-actin (lower panel) antibodies. (B) Relative expression of D1 and TRβ1 mRNA in HepG2 cells. Medium control (solid bars), IL-1β stimulation for 4 h, IL-1β stimulation for 4 h with preincubation for 30 min of 20 or 50 μM SP600125 or vehicle DMSO. Mean values ± S.E.M. (n=4) are shown. Significances were evaluated by Mann-Whitney U-tests; *P≤0.05.

SP600125, a specific AP-1 inhibitor, was used. HepG2 cells were preincubated for 30 min 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 h of IL-1β stimulation RNA was isolated and D1 and TRβ1 mRNA was measured. No difference was observed between the control stimulations with the vehicle DMSO and the stimulations with SP600125 (Fig. 2B).

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. Thirty minutes before IL-1β stimulation HepG2 cells were incubated with 2 and 3 mM sulfasalazine. Protein analysis showed that after preincubation with 3 mM sulfasalazine, IL-1β stimulation did not result in

phosphorylation of IκBα in the sulfasalazine-treated cells compared with the vehicle DMSO control (Fig. 3A), which showed that the NFκB pathway was indeed inhibited. After 4 h of IL-1β stimulation, RNA was isolated. mRNA analysis showed that the TRβ1 mRNA decrease was abolished by 2 and 3 mM sulfasalazine compared with 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 preincubated for 30 min with 50 μM SP600125 and 3 mM sulfasalazine. Cells were subsequently stimulated with IL-1β for 4 h. RNA was isolated

Figure 3 (A) Protein expression in HepG2 cells after 0, 15, 30, 60 and 120 min of IL-1β stimulation, following preincubation for 30 min 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 (solid bars), IL-1β stimulation for 4 h, IL-1β stimulation for 4 h with preincubation for 30 min with 2 or 3 mM sulfasalazine or vehicle DMSO. Mean values ± S.E.M. (n=6) are shown. Significances were evaluated by Mann-Whitney U-tests; *P≤0.05, **P≤0.01.

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 pathways, as is shown in Fig. 4 (P=0.004 for D1 and TRβ1).

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 the liver of mice after LPS administration, an animal model of (acute) non-thyroidal illness. 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 24 h of IL-1β stimulation, which is in agreement with the study of Jakobs *et al.* (2002). The TRβ1 mRNA decrease did not, however, result in a decrease of TRβ1 protein levels in contrast to the *in vivo* studies by Beigneux *et al.* (2000). 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. Other post-translational events, induced by LPS or other cytokines,

Figure 4 Relative expression of D1 and TRβ1 mRNA in HepG2 cells. Medium control (solid bars), IL-1β stimulation for 4 h (open bars), IL-1β stimulation for 4 h with preincubation for 30 min of 3 mM sulfasalazine and 50 μM SP600125 (light grey bars) or vehicle DMSO (darker grey bars). Mean values ± S.E.M. (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.

are probably necessary to decrease the amount of TRβ1 protein. We specified the influence of NFκB on AP-1 activation by using the specific AP-1 inhibitor SP600125 and the specific NFκB inhibitor sulfasalazine separately and simultaneously. Sulfasalazine prevented IκBα 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 was 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 analysis showed that there are three possible NFκB-binding sites present in the TRβ1 promoter (Schug & Overton 1998). It is possible that these sites are responsible for the NFκB-mediated transcriptional repression of the TRβ1 gene. mRNA degradation during inflammation has been reported for Connexin 32, 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.* (2000) 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. A schematic overview of these mechanisms is shown in Fig. 5A. It can be hypothesized that reduced levels of TRβ1 mRNA and subsequently reduced 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 the TRβ1 protein levels of HepG2 cells after IL-1β stimulation are not reduced compared with 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.* (2000). 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 & Koenig (2000) showed that the IL-1β-induced decrease of D1 gene transcription in rat hepatocytes can be partially overcome by adding exogenous SRC-1. Yu & Koenig (2000) therefore hypothesized that limiting the amounts of coactivators could play a role

in the cytokine-induced decrease of D1 mRNA. This hypothesis is supported by the study of Jakobs *et al.* (2002), 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. 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 was 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 Fig. 5B.

In summary, the IL-1β-induced D1 and TRβ1 mRNA decreases 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 the transcriptional activation of NFκB-, AP-1- and TRβ1-mediated D1 gene transcription.

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