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EXPERIMENTAL STUDY

Proinflammatory cytokines inhibit the expression and function of human type I 5′-deiodinase in HepG2 hepatocarcinoma cells

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Abstract

Objective: The sick euthyroid syndrome in critically ill patients without primary disease of the thyroid gland is characterised by low serum total triiodothyronine (T_3), normal to elevated thyroxine (T_4), elevated reverse T_3 (rT_3) and normal TSH levels. The aim of this work was to clarify if impaired T_4 and rT_3 5′-deiodination is an underlying mechanism.
Design and Methods: We analysed the effect of the human recombinant proinflammatory cytokines interleukin (IL)-6 and IL-1β, tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) on human type I 5′-iodothyronine deiodinase (5′DI) enzyme activity in the human hepatocarcinoma cell line HepG2, i.e. in a homologous human system. Furthermore, we analysed transcriptional effects of the cytokines by transient transfection assays using the luciferase or chloramphenicol acetyltransferase (CAT) reporter genes under the control of 1480 nucleotides of the human 5′DI promoter.
Results: IL-6 at 500 pg/ml and TNF-α at 25 ng/ml had no significant effect, whereas 100 ng/ml IFN-γ or 10 ng/ml IL-1β reduced 5′DI enzyme activity to 77.9 and 59.5% of control values. IFN-γ did not alter, IL-6 and TNF-α moderately decreased (in the case of IL-6 only in the CAT system), and IL-1β (0.01–10 ng/ml) dose-dependently inhibited 5′DI promoter activity to a minimum of 38.1%.
Conclusion: IL-1β inhibited both 5′DI enzyme and promoter activity and, thus, may exert its effect on thyroid hormone metabolism at least partially through direct inhibition of hepatic 5′DI gene transcription.

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Introduction

The terms low triiodothyronine (T_3) syndrome or sick euthyroid syndrome (SES) refer to the changes in serum thyroid hormone status that are observed in critically ill patients without primary involvement of the thyroid gland itself. SES is characterised by a decrease in serum total T_3 levels and normal to elevated thyroxine (T_4) levels, accompanied by a variable rise in serum reverse T_3 (rT_3), whereas thyrotrophin (TSH), at least in the less severe cases, remains unchanged. Whether this condition is a physiological response serving to decrease the energy expenditure and hence the catabolism of the diseased organism, or a maladaptation that, by producing tissue hypothyroidism, aggravates the underlying illness, has not unequivocally been answered (1–3). It is documented that in a variety of diseases, such as trauma, cardiovascular disease, sepsis or extensive surgery, the occurrence of SES is a negative prognostic marker, especially in the context of low T_4 and/or TSH levels (4–6). However, no clear-cut benefit from treatment of SES with thyroid hormones has yet been demonstrated (for review, see (7, 8) and references therein).

The pathogenesis of SES is incompletely understood. In humans, most of the circulating T_3 is generated by the 5′-deiodination of T_4 through type I 5′deiodinase (5′DI). This enzyme is a selenoprotein located mainly in the liver, kidney, pituitary and thyroid gland (9). As the main findings in SES are a decrease in serum T_3 levels accompanied by elevated serum rT_3, an impairment of hepatic 5′-deiodination of T_4 and rT_3 by 5′DI has been implicated and proposed to be a part of the acute phase response to injury and infection. The acute phase reaction is initiated by the action of proinflammatory cytokines, especially interleukin (IL)-6 and -1 and tumour necrosis factor-α (TNF-α) (10–12). It has therefore been speculated that cytokines

may directly interfere with the 5′-deiodination of T_4. A reduction of 5′DI mRNA levels by IL-1β, TNF-α and interferon-γ (IFN-γ) has been observed in the rat thyroidal cell line FRTL-5 (13) and IL-6 also has been found to be effective in the same model (14). In addition, IL-6 knock-out mice are partially, albeit not fully, protected against development of the SES in response to experimentally induced illness (15). In contrast, others report an increase, rather than a decrease, of 5′DI activity or mRNA levels in response to IL-1β in mice (16) and to IL-1β, IL-6 and TNF-α in rat phi 1 liver cells (17). In reaggregate cultures of the rat pituitary gland, IL-1β stimulated 5′DI and 5′DII activity and TNF-α stimulated 5′DII activity. *In vivo*, lipopolysaccharide (LPS) injection into male adult rats stimulated both deiodinases in the anterior pituitary, concomitant with a decrease of serum TSH levels (18).

To further clarify the issue whether inflammatory cytokines directly regulate 5′DI expression or modulate the enzyme activity, we have studied the effects of IL-1β, IL-6, TNF-α and IFN-γ on the human hepatocarcinoma cell line HepG2. This cell line was chosen because it expresses the receptors for the above mentioned cytokines (19, 20) and also exhibits significant 5′DI activity. We performed both transient transfection of the human 5′DI promoter and enhancer region fused to luciferase or chloramphenicol acetyltransferase (CAT) reporter genes to directly measure cytokine effects on 5′DI gene expression and a 5′DI enzyme assay to determine 5′DI activity in cytokine-treated HepG2 cells. This human cell line together with the human recombinant cytokines and the human 5′DI promoter represents an entirely homologous system which should exclude any effects of species differences. We demonstrate that, except for IL-6, all cytokines tested decrease 5′DI enzyme and/or promoter activity, TNF-α and IFN-γ only slightly, IL-1β being the most potent.

Materials and methods

Materials

[^{125}I]rT_3 was purchased from DuPont (Nemours, Belgium). Human recombinant cytokines were obtained from Strathmann Biotec AG (Hamburg, Germany) and dissolved in sterile Hanks' balanced salts solution (HBSS). Cell culture media and additives were obtained from Life Technologies (Eggenstein, Germany).

Plasmids for reporter assays

Human 5′DI promoter constructs contained 1480 bp from the 5′ upstream regulatory region of the human 5′DI gene fused to the luciferase or CAT reporter gene in the vectors pGL2 and pCAT respectively, purchased from Promega (Madison, WI, USA). Preparation of these constructs was described in detail elsewhere (21). Plasmids were purified using a kit from Qiagen (Hilden, Germany) according to the manufacturer's instructions.

Cell culture and transfection

HepG2 cells (ATCC, HB 8065) were cultured in Dulbecco's modified Eagle's medium-F12 (DMEM-F12) with 10% fetal calf serum (FCS) and 1 mg/ml L-glutamine in 75 cm^2 flasks, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. For transfection, the cells were seeded in 6- or 12-well plates and grown overnight. Then, the medium was replaced with fresh DMEM-F12 without FCS and the cells were incubated for additional 48 h. Transfections were carried out at a cell density of 50–70% using Lipofectamine (Life Technologies) for liposome-mediated gene transfer (22). Ten microlitres of Lipofectamine and 1 μg of the reporter plasmids were used per well in the case of 6-well plates, 5 μl Lipofectamine and 0.5 μg of plasmid for 12-well plates. For internal control of the transfection efficiency, 1 μg (0.5 μg for 12-well plates) of a plasmid expressing the gene for bacterial β-galactosidase under the control of an SV40 promoter was cotransfected. After an incubation period of 24 h, the cells were washed briefly and stimulated once with serum-free DMEM-F12 containing IL-1β, IL-6, TNF-α or IFN-γ in varying concentrations; the controls received DMEM-F12 alone. For kinetic studies, cells were harvested after 3, 6, 12 and 24 h; for all other experiments, cells were harvested at 24 h. Luciferase and β-galactosidase activities were measured using the reagent kits supplied by Promega, and CAT activities were determined with an ELISA kit from Boehringer (Mannheim, Germany), according to the suppliers' protocols. Luciferase and CAT activities were normalised to transfection efficiency as determined by measurement of β-galactosidase and expressed as per cent of (untreated) control values.

5′DI assay

Cells were seeded in 6-well plates in DMEM-F12 with 10% FCS and grown overnight. Then, the FCS was withdrawn and cells were incubated with DMEM-F12 containing 100 nmol/l sodium selenite without or with cytokines in varying concentrations for dose–response experiments; a single dose of cytokines was applied. Incubation was carried out for 24, 48, 72 and 96 h for kinetic studies. For the 5′DI assay, the cells were washed twice with cold HBSS and homogenised in a buffer containing 250 mmol/l glucose, 20 mmol/l Hepes, 1 mmol/l EDTA and 1 mmol/l dithiothreitol by ultrasonication (ten pulses of 0.5 s at 200 W). The protein contents of the homogenates were measured by a modified Bradford assay (Bio-Rad, Munich, Germany). Specific activities of 5′DI in the homogenates were determined measuring the release of $^{125}I^-$ from [^{125}I]rT_3 in the presence of 100 nmol/l

unlabelled rT_3; the incubation volume was 50 µl and the incubation time was 1 h. In some assays, the 5′DI inhibitor 6-propyl-2-thiouracil was used to test for specificity (23). All samples were measured in duplicate using 20 µg protein and 5′DI activity was expressed as pmol $^{125}I^-$ release/mg protein per min.

Statistical analysis

5′DI enzyme assays were done seven times in duplicates and transfection experiments were done three times in triplicates. Results are presented as the means±S.E.M. of all experiments. Statistical analysis was performed using the SPSS program package (SPSS Inc., Chicago, IL, USA). Data were analysed by a Kruskal–Wallis test with the cytokine treatment as grouping factor. When the main effect was found to be significant ($P < 0.05$) or showed a tendency to significance ($P < 0.1$), data from separate cytokine treatments were compared with the controls by a Mann–Whitney U test. $P < 0.05$ was considered significant.

Results

Cytokine effects on 5′DI activity in HepG2 cells

Optimal conditions for measuring cytokine effects on 5′DI enzyme activity were determined by dose-response and kinetic studies. HepG2 cells were incubated for 48 and 72 h with IL-6 at 10, 100, 1000 and 5000 U/ml; with TNF-α at 0.5, 5, 25 and 100 ng/ml; with IFN-γ at 1, 10, 100 and 500 ng/ml; and with IL-1β at 0.01, 0.1, 1, 10 and 100 ng/ml. Treatments caused either a more or less marked decrease of 5′DI activity or no effect. Concentrations that produced the most prominent effect without exceeding the dosage recommendation of the manufacturer were chosen for further experiments: these were 100 U/ml, equivalent to 500 pg/ml, IL-6; 25 ng/ml TNF-α; 100 ng/ml IFN-γ; and 10 ng/ml IL-1β. A significant dose response was seen only in the case of IL-1β (see below). In a more extended kinetic study, the optimal time for cytokine treatment was tested. HepG2 cells were incubated for 24, 48, 72 and 96 h with the respective cytokines at the concentrations mentioned before. Any observed effects were most prominent at 48 h and declined again at 72 and 96 h (data not shown). For further experiments, cells were harvested at 48 h.

5′DI activity in extracts of HepG2 cells was measured in seven experiments with duplicate determinations. Basal specific activity was 7.07± 0.9 pmol/min per mg (±S.E.M.) of iodide release from 100 nmol/l rT_3 (Fig. 1). This value was used as control (100±6.2%); all other enzyme activities are expressed as per cent of the activity measured in untreated control cells. Stimulation of HepG2 cells with 500 pg/ml IL-6 or 25 ng/ml TNF-α for 48 h did not alter enzyme activity significantly. In contrast, a significant inhibition of 5′DI activity to 77.9±6.1% ($P < 0.01$) of control values was observed after incubation of the cells with 100 ng/ml IFN-γ for 48 h. IL-1β dose-dependently inhibited 5′DI enzyme activity to 75.7±4.9% at 1 ng/ml ($P < 0.01$) and 59.5±3.8% at 10 ng/ml IL-1β ($P < 0.001$) (Fig. 1).

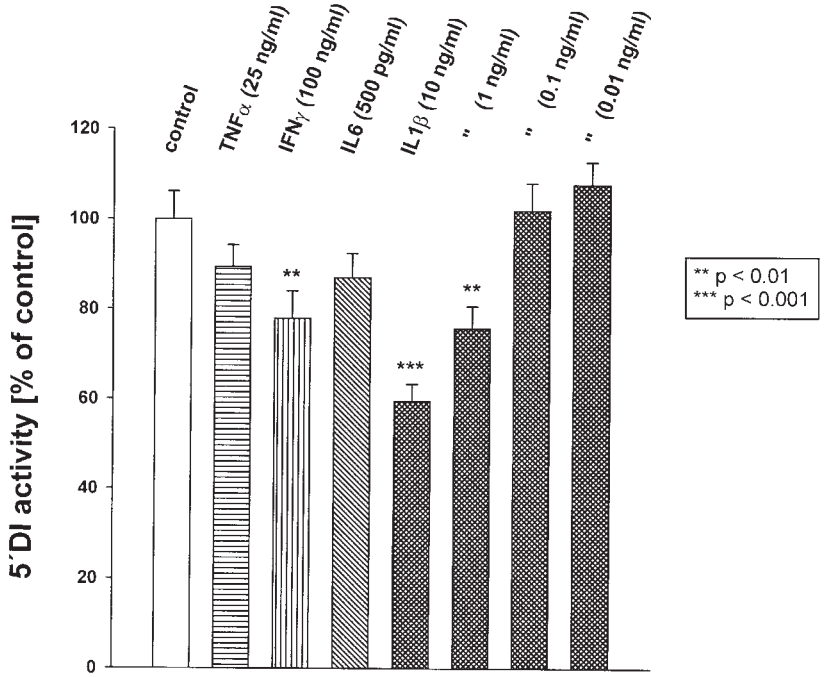

Figure 1 Cytokine effects on 5′DI enzyme activity in HepG2 cells. Shown is the 5′DI activity measured in HepG2 cells treated with the cytokines indicated and in untreated control cells. Bars show means±S.E.M. of seven experiments done in duplicate; *P*-values vs control by Mann–Whitney U test.

Cytokine effects on 5′DI promoter-driven luciferase and CAT activities

To test whether the cytokine effects on 5′DI activity were due to a direct modulation of 5′DI promoter activity, transient transfection studies were performed. A 1480 bp fragment of the 5′DI upstream promoter and enhancer region was fused to the luciferase reporter gene and transfected into HepG2 cells. The pGL2 basic vector, carrying a luciferase reporter gene without a promoter, was used to determine the background luciferase activity. None of the cytokines had a significant effect on the basal luciferase activity derived from the 'empty' reporter vector pGL2 (data not shown).

Dose-response experiments were performed to determine the best cytokine concentrations for 5′DI promoter studies (not shown). The same concentrations as used in the 5′DI activity assay were found optimal and were used during transfection experiments. Kinetic studies revealed that cytokine effects on 5′DI promoter-driven luciferase activity, if any, were strongest at 24 h. An exception was IL-1β, where an inhibition was already seen at 3 and 6 h, but did not get more prominent at 12 or 24 h as compared with 6 h (Fig. 2). Longer incubation was not performed considering the limited half-life of the luciferase enzyme. Therefore, in further experiments, cells treated with the different cytokines were harvested together at 24 h for convenience.

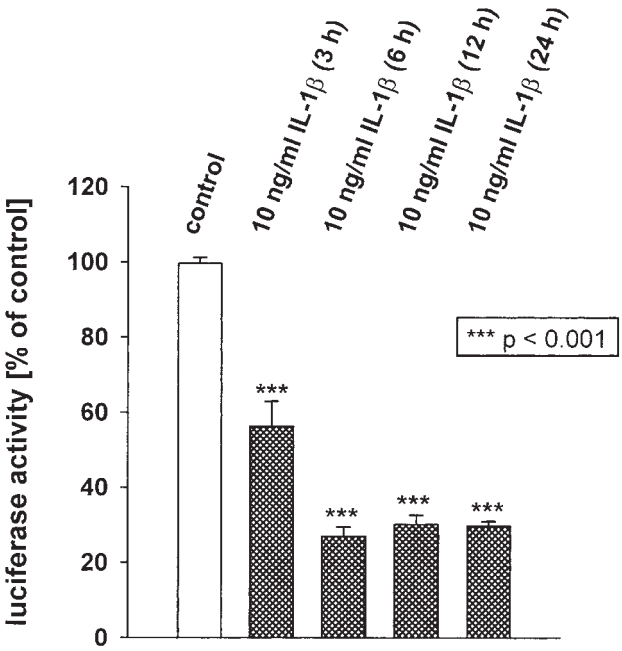

Figure 2 Time course of IL-1β-dependent inhibition of 5′DI promoter activity. HepG2 cells were transiently transfected with the luciferase reporter construct of the 5′DI promoter. Afterwards they were treated with 10 ng/ml IL-1β for the time intervals indicated. Transfected cells left untreated served as a control. Bars show means±S.E.M. of three experiments done in triplicate; *P*-values vs control by Mann–Whitney U test.

5′DI promoter-driven luciferase activity in control HepG2 was set as 100±4.9% (see Fig. 3a). TNF-α at 25 ng/ml inhibited 5′DI promoter activity significantly to 63.8±2.5% ($P < 0.001$). Even the lowest concentration of IL-1β, 0.01 ng/ml, reduced 5′DI promoter activity to 64.6±14.4% ($P < 0.05$). Higher concentrations (0.1–10 ng/ml) further reduced luciferase activities to a minimum of 38.1±3.2% at 10 ng/ml ($P < 0.001$). Higher concentrations of 50 or 100 ng/ml had no stronger effect (data not shown). Alterations triggered by 500 pg/ml IL-6 or 100 ng/ml IFN-γ were not significant. A total of three luciferase assays with triplicate determinations was performed in each case.

To control for effects that may be caused by cryptic response elements in the luciferase vector backbone, the 1480 bp fragment of the 5′DI promoter and enhancer region was also fused to the CAT gene and transfected into HepG2 cells. After transfection, the cells were treated with the same cytokine concentrations as above ($n = 3$, triplicate determinations). CAT activity of the control cells receiving no cytokine treatment after transfection was set as 100±10.8% (Fig 3b). IL-6 at 500 pg/ml reduced 5′DI promoter-driven CAT activity to 57.1±8.2% of control values ($P < 0.05$). As a tendency, 25 ng/ml TNF-α and 100 ng/ml IFN-γ reduced promoter activity (70.7±7.1 and 75.3±14.3%), although these effects failed to reach significance. The lowest tested concentration of IL-1β (0.01 ng/ml) had no effect on CAT activity. Higher concentrations (0.1, 1 and 10 ng/ml) inhibited 5′DI promoter-driven CAT activity to 64.9±5.5, 37.0±3.2 and 40.5±4.6% respectively ($P < 0.01$).

Cotransfection of a β-galactosidase expression vector and determination of β-galactosidase activity was used to calibrate luciferase activities with respect to transfection efficiency. As β-galactosidase was sensitive to IL-1β (not to the other cytokines), responding with a dose-dependent increase in enzyme activity, calibration resulted in a slight exaggeration of the effects. Therefore, reporter activities were also normalised to protein content of the samples. In this case, IL-1β-triggered reduction in luciferase activity was 42.3±4.9% for 1 ng/ml IL-1β and 56.1±4.4% for 10 ng/ml IL-1β in the luciferase assay, and 48.4±7.9% for 1 ng/ml IL-1β and 48.9±6.1% for 10 ng/ml IL-1β in the CAT assay. These effects were also significant ($P < 0.01$) (Fig. 3c).

Discussion

The acute phase is the response of the organism to severe pathological conditions such as infection, trauma, neoplastic growth or immunological diseases. It is usually thought of as a physiological reaction

intended to restore homeostasis. Cytokines may produce SES on the level of the thyroid or the pituitary (1); however, one of their main target organs is the liver, and recognisable alterations of hepatic protein synthesis occur during the acute phase (24). In this context, especially IL-1β, IL-6, TNF-α and IFNs are of interest as both positive and negative regulators (25, 26). As SES has been proposed to be part of this acute phase reaction, it was asked whether the aforementioned cytokines play a role in its pathogenesis.

Both decreased serum T_3 levels and increased IL levels have been correlated with the severity of the underlying disease. It was demonstrated that IL-6 levels >20 pg/ml (normal ≤ 5 pg/ml) in patients with breast cancer suggest a poor prognosis (27). Similar observations were reported for renal carcinoma (28, 29). Also, in postoperative patients, strongly elevated IL-6 levels (>400 pg/ml) are predictive of complications (30). A negative correlation between IL-6 and T_3 serum levels has been observed clinically even at mildly elevated IL-6 levels (>15 pg/ml) (31). IL-1β also has been implicated in the pathogenesis of SES. Infusion of IL-1β produces a similar picture in experimental rats (32) and was shown to reduce hepatic 5′DI mRNA in mice (33). TNF-α levels have also been investigated in patients with SES, but were found to be within the normal range (34).

Even though cytokine effects on 5′-deiodination have been studied at the level of 5′DI gene expression and enzyme activity, the results have not been unambiguous. Whereas one group reports a stimulation of hepatic 5′DI mRNA by TNF-α, IL-1β and IL-6 in rat phi1 cells (17), others find a reduction by these cytokines in rat FRTL-5 thyroid cells (13). This may reflect inter-species variation or effects obtained in heterologous test models. However, tissue-specific differences in cytokine effects on deiodinase activities may be expected according to distinct roles of deiodinases in specific tissues; for example, in reaggregate cultures of the rat pituitary gland, Il-1β stimulated 5′DI activity and TNF-α stimulated 5′DII activity. An increase of 5′DI activity in the anterior pituitary concomitant with a decrease of serum TSH levels is triggered by LPS injection into male adult rats. This suggests a role of cytokine-induced stimulation of T_3 production in the pituitary as part of the disturbed negative feedback regulation preventing an increase in TSH levels observed in severe cases of SES (18).

We have sought to avoid ambiguity due to species-specific differences in the effect of cytokines on 5′DI gene transcription and enzyme activity by using a homologous system with the human cell line HepG2, human recombinant cytokines and the human 5′DI promoter region for the transient transfection assays. HepG2 cells are a highly differentiated hepatocarcinoma cell line that contain endogenous 5′DI activity and can be grown under serum-free conditions for up to 4 days with selenium supplementation. Serum-free medium was chosen for the experiments as FCS may contain unspecified amounts of cytokines, growth factors or their binding proteins, which would interfere with the experimental set-up.

IL-6 and TNF-α did not have a significant effect on 5′ enzyme activity in this cell culture model even at cytokine concentrations above the range commonly observed in patients with SES. In contrast, IFN-γ and IL-1β did inhibit enzyme activity. Normal IL-1β serum levels are up to 60 pg/ml and inhibition of 5′DI activity was seen at a concentration of 1 ng/ml, which may rarely be reached in the serum. The liver, however, contains tissue macrophages capable of secreting cytokines, so their concentration in the microenvironment of the liver cells may be considerably higher and sufficient for inhibition of 5′DI. Arguing against this is the finding that hepatic macrophage depletion does not prevent LPS-induced SES in experimental mice (35). Given the profound effects of IL-1β on thyrocytes (36) or pituitary cells (18), similar considerations may apply to 5′DI regulation by this cytokine in the thyroid or pituitary glands. Our results revealing a lack of significant inhibition of 5′DI activity by TNF-α correspond with the data published by Nagaya *et al.* (37). They found a reduction of T_3-stimulated, but not basal levels, of 5′DI activity in HepG2 cells, which they ascribed to an indirect effect of nuclear factor kappa B on the transcription of T_3-regulated genes.

To test whether the cytokines directly influence transcriptional activity of the human 5′DI promoter in a human cell line, transient transfection assays were performed. Both luciferase and CAT were used as reporter genes. We could not demonstrate an effect of IL-6 on 5′DI promoter activity using luciferase as the reporter gene, but a significant inhibition was detected with CAT. This may be due to cryptic regulatory elements that have been observed in reporter vector backbones, which may trigger independent responses to treatment which can bias the result from the cloned promoter sequence. Alternatively, cytokines may have variant effects on different reporter enzymes at the pre- or post-translational levels. As a consequence, we regard as substantial only those results where both reporter assays and the enzyme activity demonstrate comparable responses to a certain treatment, which is the case for IL-1β (see below). IFN-γ had no significant effect in either the luciferase or CAT assays.

TNF-α reduced 5′DI promoter activity, even though the effect was significant only when the luciferase reporter gene system was used. Nagaya *et al.* (37) described similar results, although a reduction in 5′DI promoter-driven luciferase activity was measurable in their case only after stimulation with T_3. At the lowest concentration tested, IL-1β inhibited 5′DI promoter-driven luciferase activity slightly, but not in the CAT assay. In both assays, however, higher concentrations of IL-1β decreased 5′DI promoter activity. These data are in principle in agreement with the

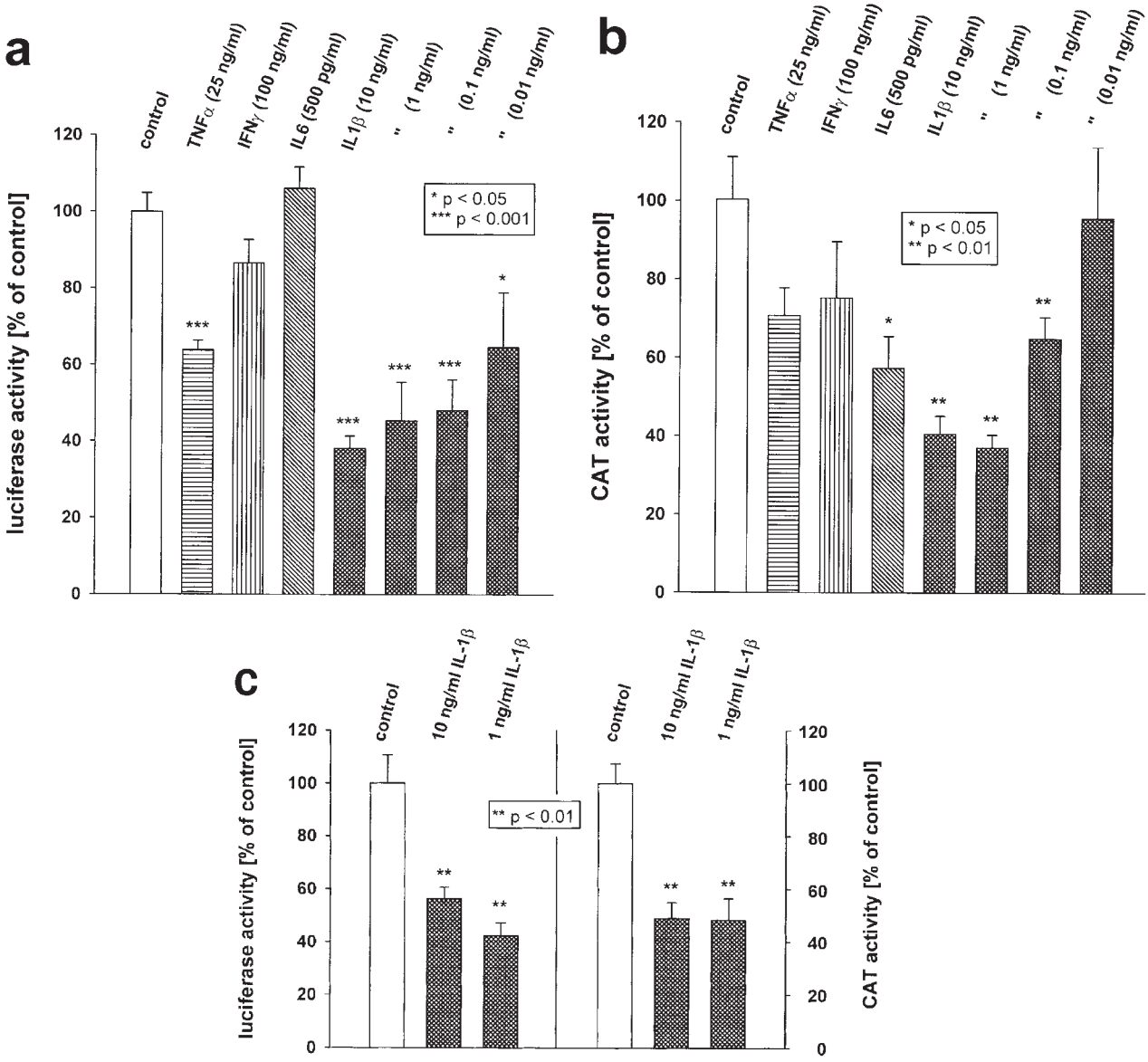

Figure 3 Cytokine effects on 5′DI promoter-driven luciferase and CAT activities. HepG2 cells were transiently transfected with luciferase or CAT reporter constructs of the 5′DI promoter. Afterwards they were treated with the cytokines indicated or left untreated as a control. (a) Luciferase activities normalised to β-galactosidase activity; (b) CAT activities normalised to β-galactosidase activity; (c) luciferase and CAT activities normalised to the protein contents of the samples. Bars show means±S.E.M. of three experiments done in triplicate; *P*-values vs control by Mann–Whitney U test.

enzyme activity assay. The concentration needed to inhibit 5′DI promoter-dependent transcription was at least 10-fold lower than that needed to achieve enzyme activity reduction. This is probably due to the long half-life of the 5′DI enzyme. Using another model system, primary rat hepatocyte cultures, Yu & Koenig (38) recently postulated cytokine-induced competition for limiting amounts of the transcriptional coactivator SRC-1 as a reason for decreased transcription of the 5′DI gene in non-thyroidal illness. Rowan *et al.* (39) reported that SRC-1 is phosphorylated in response to signalling via the MAPK pathway, which is also activated by cytokines such as TNF-α and IL-1 (40). Thus, phosphorylation may be another regulatory mechanism accounting for cytokine effects on SRC-1 activity. By RT-PCR, expression of SRC-1 was demonstrated in HepG2 cells (G Möller & C Schmutzler, unpublished observations), suggesting that regulation of 5′DI expression by cytokines may involve this transcriptional coactivator also in this cell line.

In summary, we did not observe conclusive effects of IL-6, TNF-α or IFN-γ on 5′DI enzyme activity or on its

promoter. For IL-6 this is somewhat surprising. Serum levels of this cytokine have been demonstrated to correlate well with the severity of pathological conditions and thus SES. However, IL-6 effects on thyroid hormone metabolism need not necessarily be direct. IL-6 is known to induce a variety of factors, including other cytokines, cytokine receptors and cytokine-binding proteins (41). The influence of IL-6 on thyroid hormone metabolism is further complicated by the fact that this cytokine decreases the transcription of genes encoding thyroid hormone-binding proteins (42), making its *in vivo* effects difficult to reproduce in *in vitro* models. Furthermore, certain cytokine effects in the liver may be mediated by accessory cells like the stellate cells, which respond to and secrete cytokines themselves. In the pituitary, a similar role may be played by the folliculo-stellate cells (18).

In contrast, IL-1β clearly inhibited 5′DI enzyme activity and 5′DI promoter-driven transcription. The observation of an SES after infusion of IL-1β may therefore at least in part be explained by a direct down-regulation of hepatic 5′DI at the transcriptional level.

Acknowledgements

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