EXPERIMENTAL STUDY

Interleukin-18, a proinflammatory cytokine, contributes to the pathogenesis of non-thyroidal illness mainly via the central part of the hypothalamus-pituitary-thyroid axis

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Abstract

Objective: Proinflammatory cytokines are involved in the pathogenesis of non-thyroidal illness (NTI), as shown by studies with IL-6$^{-/-}$ and IL-12$^{-/-}$ mice. Interleukin (IL)-6 changes peripheral thyroid hormone metabolism, and IL-12 seems to be involved in the regulation of the central part of the hypothalamic-pituitary-thyroid (HPT) axis during illness. IL-18 is a proinflammatory cytokine which shares important biological properties with IL-12, such as interferon (IFN)-γ-inducing activity.

Design: By studying the changes in the HPT-axis during bacterial lipopolysaccharide (LPS)-induced illness in IL-18$^{-/-}$, IFNγR$^{-/-}$ and wild-type (WT) mice, we wanted to unravel the putative role of IL-18 and IFNγ in the pathogenesis of NTI.

Results: LPS induced a decrease in pituitary type 1 deiodinase (D1) activity ($P < 0.05$, ANOVA) in WT mice, but not in IL-18$^{-/-}$ mice, while the decrease in D2 activity was similar in both strains. LPS decreased serum thyroid hormone levels and liver D1 mRNA within 24 h similarly in IL-18$^{-/-}$, and WT mice. The expression of IL-1, IL-6 and IFNγ mRNA expression was significantly lower in IL-18$^{-/-}$ mice than in WT, while IL-12 mRNA expression was similar. IFNγR$^{-/-}$ mice had higher basal D1 activity in the pituitary than WT mice ($P < 0.05$); LPS induced a decrease of D2, but not of D1, activity in the pituitary which was similar in both strains. In the liver, the LPS-induced increase in cytokine expression was not different between IFNγR$^{-/-}$ mice and WT mice, and the decrease in serum T_3 and T_4 levels and hepatic D1 mRNA was also similar.

Conclusions: The relative decrease in serum T_3 and T_4 and liver D1 mRNA in response to LPS is similar in IL-18$^{-/-}$, IFNγR$^{-/-}$ and WT mice despite significant changes in hepatic cytokine induction. However, the LPS-induced decrease in D1 activity in the pituitary of WT mice is absent in IL-18$^{-/-}$ mice; in contrast, LPS did not decrease pituitary D1 activity in the IFNγR$^{-/-}$ mice or their WT, which might be due to the genetic background of the mice. Our results suggest that IL-18 is also involved in the regulation of the central part of the HPT axis during illness.

European Journal of Endocrinology **151** 497–502

Introduction

Changes in thyroid hormone metabolism are frequently observed in ill patients without primary thyroid diseases. These changes, also known as non-thyroidal illness (NTI) or sick euthyroid syndrome (SES), are characterized by a decrease of serum T_3 and an increase in serum rT_3 (mediated by inhibition of 5′-deiodinase (D1) and an increase in type 3 5-deiodinase (D3)), and a decrease of serum T_4 and fT_4 in severe cases. Despite these changes, serum TSH does not increase and can even be decreased. Recently, we have shown that both the peripheral and central parts of the hypothalamic-pituitary-thyroid (HPT) axis are downregulated during the acute phase of illness (1).

Proinflammatory cytokines, especially interleukin (IL)-6, IL-1β, tumour necrosis factor (TNF)-α and interferon (IFN)-γ, are important mediators of the acute phase of illness and have inhibiting effects on peripheral thyroid hormone metabolism (2). Furthermore, we have shown in an *in vivo* NTI animal model (mice receiving a sublethal dosis of bacterial endotoxin lipopolysaccharide (LPS), resulting within 24 h in decreased serum T_3 and T_4 levels and reduced liver D1 mRNA expression (3), that IL-12 might be involved in changes in the central part (pituitary D1 and D2 activity) of the HPT axis during illness (4).

IL-12 is an important proinflammatory cytokine, synergizing in its action with IL-18, another proinflammatory cytokine. IL-18 was originally identified as

IFNγ-inducing factor (IGIF). IL-18 mRNA is expressed in a wide variety of cells, including hepatic Kupffer cells, macrophages, T cells, B cells, dendritic cells, astrocytes and microglia cells. IL-18 shares biological properties with IL-12 but is not structurally related to this cytokine. IL-18 has a primary and three-dimensional structure which resembles that of IL-1 and the IL-18 receptor system, and its signal-transduction pathway is analogous to those of the IL-1 receptor, making IL-18 part of the IL-1 family (5).

We showed that the LPS-induced decrease of D1 activity in the pituitary of female mice was not present in IL-12$^{-/-}$ mice, indicating a role of IL-12 in alterations of the central part of the HPT axis during illness. However, it is unknown which IL-12-dependent factor is responsible for the observed alterations in the pituitary. IL-12 is required for IFNγ expression, which exerts inhibitory effects on different aspects of thyroid hormone metabolism (3). IL-12 is known to act synergistically with IL-18 to stimulate cellular IFNγ production, but by different signalling pathways. IL-18 activates NF-κB and AP-1, while IL-12-dependent gene expression is mediated by signalling through Stat4 and Stat3 (6). Employing IL-18$^{-/-}$ mice enables us to clarify the role of proinflammatory cytokines, especially with respect to signal-transduction pathways, in the pathogenesis of NTI. Therefore, we studied the HPT axis in our NTI model both centrally (pituitary) and peripherally (liver) in IL-18$^{-/-}$ and IFNγR$^{-/-}$ mice compared to wild-type (WT) animals. By studying the HPT axis during systemic illness in IFNγR$^{-/-}$ mice, we are able to exclude the effects of IL-18-induced IFNγ on altered thyroid hormone metabolism.

Materials and methods

Animals

IL-18$^{-/-}$ mice (from a C57/Bl6 background) were generated by Takeda and colleagues (7). Normal C57BL/6 WT mice, used as controls for IL-18$^{-/-}$ mice, were obtained from Harlan Sprague–Dawley (Horst, The Netherlands). IFNγR$^{-/-}$ mice (from a 129/Sv/Ev genetic background (8)) and 129/Sv/Ev WT mice were donated by M Kopf (Basel Institute for Immunology). Female IL-18$^{-/-}$, IFNγR$^{-/-}$ and matching WT mice were used at 6–12 weeks of age. The mice were kept in 12-h light/dark cycles in a temperature-controlled room (22 °C) with food and water *ad libitum*. A week before the experiment, the mice were housed in groups according to the experimental set-up.

Mice were injected intraperitoneally with 150 g LPS (endotoxin, *E. coli* 127:B8, Sigma), diluted in 0.5 ml sterile 0.9% NaCl. At different time points after LPS injection (t = 0, 2, 4, 8 and 24 h), six mice were anaesthetized with isoflurane and killed. Blood was taken by cardiac punction, and serum was stored at −20 °C until analysed. The liver and pituitary were obtained

and stored immediately in liquid nitrogen. The study was approved by the local animal welfare committee.

Thyroid hormones

Serum T_3 and T_4 were measured with in-house RIA (9). To prevent interassay variation, all samples of one experiment were measured within the same assay.

RNA isolation and RT-PCR

mRNA was isolated from 10 mg liver tissue with 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 First Strand cDNA synthesis kit for RT-PCR (AMV) (Roche Molecular Biochemicals, Mannheim, Germany). Real-time PCR (using the LightCycler, Roche Molecular Biochemicals, Mannheim, Germany) was performed for the quantitative estimation of D1, IL-1β, IL-6, IL-12, IL-18 and IFNγ, as described before (4). All results were corrected as to their mRNA content, using HPRT mRNA as a housekeeping gene.

D1 and D2 activity

Pituitary tissues were homogenized in ice-cold homogenization buffer (250 mM sucrose, 20 mM HEPES, 1 mM ethylendiamine tetraacetic acid and 1 mM dithiothreitol (DTT), pH 7.0) by sonication. The protein contents of the homogenates were determined by a modified Bradford protein assay (BioRad, Munich, Germany), using β-globulin as protein standard (10). Total protein of 20–60 g was used for the assays.

Specific activities of D1 and D2 in pituitary homogenates were determined in parallel in the absence or presence of 1 mM PTU (6-*n*-propyl-2-thio-uracil), using 10 nM non-radioactive rT3. The fraction of iodide release blocked by 1 mM PTU was assigned to D1. 5′-D activities of each sample were determined in triplicates and expressed as fmol ^{125}I released per min/mg protein for pituitary (11).

Statistics

Differences between cytokine knockout and WT mice after LPS administration were evaluated by analysis of variance with two grouping factors (time and treatment), using Excel (Microsoft). The differences between cytokine knockout and WT mice at different time points were analysed by Student's *t*-test (Excel).

Results

IL-18$^{-/-}$ mice

Pituitary D1 and D2 activities were determined at several time points after LPS administration. The activity is

presented (Fig. 1) as a percentage of the activity (in fmol/mg per min) determined before LPS administration (D1 = 17.4±9.9, D2 = 5.4±0.7; WT (C57/Bl6): D1 = 18.8±16.7, D2 = 7.6±1.8). LPS administration induced a significant decrease of D1 activity in the pituitary of WT mice. However, no decrease of D1 activity was observed in IL-18$^{-/-}$ mice ($P < 0.05$). The LPS-induced decrease of pituitary D2 activity was not significantly different in IL-18$^{-/-}$ mice from WT mice.

LPS administration resulted also in markedly increased expression of liver IL-1β, IL-6, IL-12 and IFNγ mRNA in both IL-18$^{-/-}$ and WT mice, peaking within 4 h. IL-12 mRNA expression was similar in IL-18$^{-/-}$ and WT mice, while IL-1β, IL-6 and IFNγ mRNA expression was significantly impaired in IL-18$^{-/-}$ mice ($P < 0.001$) compared to WT mice (Fig. 2).

Serum T$_3$ and T$_4$ and liver D1 mRNA expression decreased similarly within 24 h in both IL-18$^{-/-}$ and WT mice (Fig. 3) after LPS administration. This decrease in D1 transcript levels was not due to any diurnal variation, because liver D1 mRNA expression in saline-injected WT mice increased after 8 h (data not shown). However, the serum levels of T$_3$ and T$_4$ at the beginning of the experiment (basal levels) were significantly higher in IL-18$^{-/-}$ mice, while basal liver D1 mRNA expression seemed to be lower in IL-18$^{-/-}$ mice (Table 1).
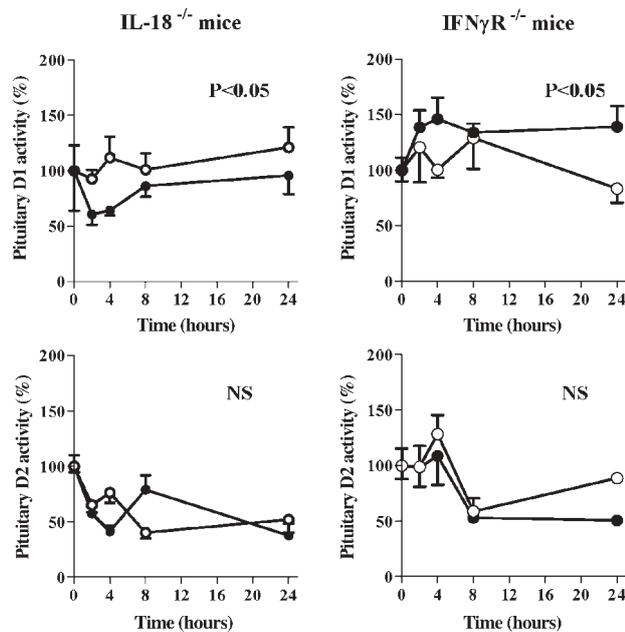
IFNγR$^{-/-}$ mice

Pituitary D1 and D2 activity in IFNγR$^{-/-}$ mice is presented (Fig. 1) as a percentage of the activity (in fmol/mg per ml) determined before LPS administration (IFNγR$^{-/-}$: D1 = 45.2±10.6, D2 = 3.8±1.4; WT (129/Sv/Ev): D1 = 21.5±5.6, D2 = 6.0±1.7). LPS administration resulted in an increase in pituitary D1 activity in 129/Sv/Ev WT mice, while no significant changes were observed in IFNγR$^{-/-}$ mice after LPS
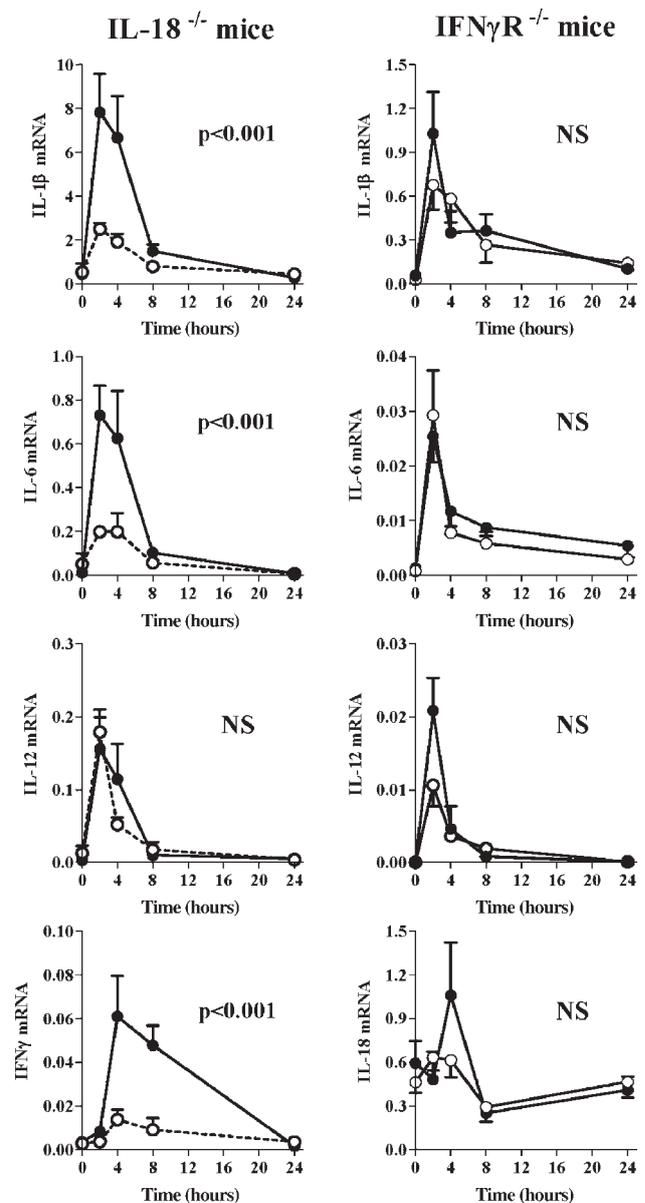

Figure 1 Relative changes of type 1 and type 2 5′-DI activity in the pituitary of IL-18$^{-/-}$ (○), IFNγR$^{-/-}$ (○) and WT (●) mice after LPS administration. Mean values±S.E.M. are depicted; *P* values indicate differences between groups by ANOVA.

Figure 2 Relative expression of liver IL-1β, IL-6, IL-12, IFNγ (only in IL-18$^{-/-}$ mice) and IL-18 (only in IFNγR$^{-/-}$) mRNA in IL-18$^{-/-}$ (○), IFNγR$^{-/-}$ (○) and WT (●) mice within 24 h after LPS administration. Mean values±S.E.M. are depicted; *P* values indicate differences between groups by ANOVA.
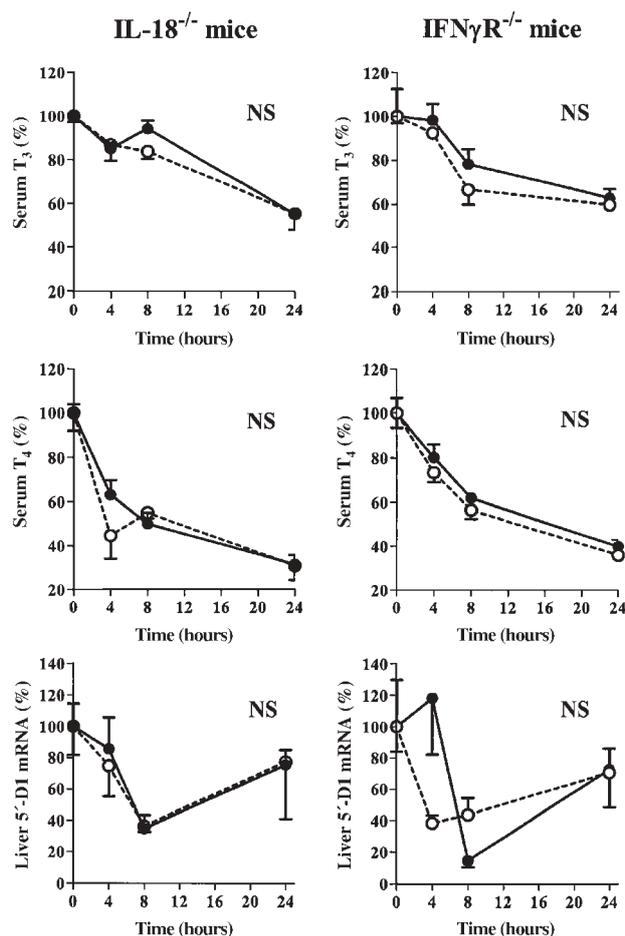
IL-18$^{-/-}$ mice

IFNγR$^{-/-}$ mice

Figure 3 Serum T_3 and T_4 levels and relative expression of liver 5'-deiodinase mRNA in IL-18$^{-/-}$ (○), IFNγR$^{-/-}$ (○) and WT (●) mice within 24 h after LPS administration. Mean values±S.E.M. are depicted; *P* values indicate differences between groups by ANOVA.

administration. However, the basal D1 activity was significantly higher in IFNγR$^{-/-}$ than WT mice. The LPS-induced decrease of pituitary D2 activity was not different in IFNγR$^{-/-}$ mice from WT mice.

Table 1 Basal thyroid hormone levels and liver D1 mRNA expression in IL-18$^{-/-}$, IFNγR$^{-/-}$ and matching strains of WT mice.

	T_3 (nmol/l)	T_4 (nmol/l)	D1 mRNA (relative expression)
IL-18$^{-/-}$	1.80±0.12**	72.5±14.1#	33.0±14.9
WT (C57B16)	1.48±0.08	58.0±5.7	43.7±15.3
IFNγR$^{-/-}$	2.45±0.19**	47.0±7.3*	0.38±0.28
WT (129SvEv)	1.57±0.47	36.6±6.3	0.66±0.24

Values were given as mean±S.D.
$P = 0.06$, IL-18$^{-/-}$ vs matching WT; *$P < 0.05$, IFNγR$^{-/-}$ vs matching WT; **$P < 0.01$, IL-18$^{-/-}$ or IFNγR$^{-/-}$ vs matching WT.

The LPS-induced cytokine response (IL-1β, IL-6, IL-12 and IL-18) in the liver did not differ in IFNγR$^{-/-}$ mice and WT mice (Fig. 2).

LPS induced a similar decrease of serum T_3, T_4 and liver D1 mRNA within 24 h in both IFNγR$^{-/-}$ and WT mice (Fig. 3), although serum levels of T_3 and T_4 at the beginning of the experiment (basal levels) were significantly higher in IFNγR$^{-/-}$ mice, while basal liver D1 mRNA expression seemed to be lower in IFNγR$^{-/-}$ mice (Table 1). LPS-induced alterations in liver D1 mRNA expression were not due to any diurnal variation, because liver D1 mRNA expression in saline-injected WT mice increased after 8 h (data not shown).

Discussion

NTI might be viewed as part of the acute-phase response, the response of an organism to severe pathological conditions, and it is predominantly mediated by IL-1-type and IL-6-type cytokines. Previously, we have shown that IL-6, an important proinflammatory cytokine, is involved in alterations of the peripheral part of the HPT axis (12). Furthermore, IL-12, another proinflammatory cytokine, might be involved in changes of the central part of the HPT axis during illness, as indicated by the absence of a LPS-induced decrease in D1 activity in the pituitary in IL-12$^{-/-}$ mice (4). IL-12 is a potent IFNγ-inducing cytokine and shares biological properties with IL-18, another proinflammatory cytokine. In the present study, we therefore evaluated the role of IL-18 and IFNγ in the pathogenesis of NTI by comparing IL-18$^{-/-}$ and IFNγR$^{-/-}$ mice with matching strains of WT mice.

We observed significant differences in basal thyroid hormone metabolism between IL-18$^{-/-}$ and IFNγR$^{-/-}$ mice and WT mice. Serum thyroid hormone levels were higher while basal liver D1 mRNA expression was lower in IL-18$^{-/-}$ and IFNγR$^{-/-}$ mice. These differences might be due to unknown genetic differences induced by genetic manipulation or to unknown inflammation-independent effects of IL-18 on basal IFNγ expression (IFNγ could interfere with several mechanisms in thyrocytes (3)), because the observed differences were present in both IL-18$^{-/-}$ and IFNγR$^{-/-}$ mice. For this reason, however, we have presented our data as percentage of decrease of basal levels (relative decrease) in order to compare the LPS-induced effects on thyroid hormone metabolism.

LPS induced peripheral and central alterations of the HPT axis. The peripheral changes, characterized by a decrease of serum T_3, T_4 and D1 mRNA expression, were similar in both IL-18$^{-/-}$ and IFNγR$^{-/-}$ mice and WT mice. We have previously shown that serum TSH remained unchanged after LPS administration compared to control animals (3). The changes in liver 5'-DI mRNA expression and serum thyroid hormones, however, were similar despite different hepatic cytokine

profiles. It is known that especially IL-1β and IFNγ might be capable of influencing 5′-DI activity or mRNA expression *in vitro* by several mechanisms (13–15), and our results suggest that the cytokines activated by LPS (relatively low levels of IL-1β, IL-6 and IFNγ in IL-18$^{-/-}$ mice) might be sufficient for the LPS-induced decrease of liver D1 mRNA.

The absence of a specific proinflammatory cytokine seems more important in the illness-induced alterations of the central part of the HPT axis. The anterior pituitary contains D1 and D2, which play a role in T_3 production and pituitary feedback (16, 17). The effects of IL-1β, IL-6 and TNFα on pituitary D1 and D2 activity in reaggregates of rat anterior pituitaries were studied, and in these reaggregates, IL-1β (dose-dependently) and TNFα increased D2 activity (18). We observed decreased pituitary D2 activity after LPS administration, which was, however, similar in IL-18$^{-/-}$, IFNγR$^{-/-}$ and WT mice.

In contrast, LPS administration did not result in decreased D1 activity in the pituitary of IL-18$^{-/-}$ mice, as was observed in matching WT mice, indicating a dominant role of IL-18 in the LPS-induced decrease of pituitary D1 activity. IL-18 exerts its action via NF-κB and AP-1 (6), and these pathways might be essential for decreasing D1 activity in the pituitary. It is known that both AP-1 and NF-κB interact with SRC-1 and CREB-binding protein (CBP), which are needed for transcription activation by NF-κB and AP-1 (19, 20). Yu and Koenig (13) recently showed that inhibition of T_3-dependent induction of D1 mRNA and activity by IL-1 and IL-6 (*in vitro*) can be partially overcome by exogenous SRC-1. It was therefore hypothesized that cytokine-induced competition for limiting amounts of coactivators decreases hepatic D1 gene expression during illness. In addition, activation of NF-κB by TNFα interferes with thyroid hormone action, as demonstrated by impairment of T_3-dependent induction of D1 gene expression in HepG2 cells (21). As mentioned before, the absence of IL-18 does not have significant effects on the LPS-induced decrease in liver D1 mRNA expression. Probably the marked increase in several hepatic cytokines, which signal also via AP-1 and NF-κB (IL-1, IL-6 and TNFα), will result in a prominent deficit of SRC-1. In the pituitary however, LPS-induced cytokine expression might be much lower; therefore, the lack of IL-18 could be critical in maintaining the balance between SRC-1 deficit (decreased D1 activity) and SRC-1 surplus (D1 activity not affected).

Furthermore, pituitary D1 regulation by T_3 occurs via TRβ2, a product of the TRβ gene and the abundant isoform in the pituitary (22), while TRβ1 is the major receptor form in liver. Recently, we have shown that LPS administration results in decreased TRβ2 mRNA expression in the pituitary (1), which might contribute to decreased D1 activity in the pituitary. Competition for limiting amounts of coactivators might also play a role in decreased pituitary TRβ2 mRNA expression, because in the pituitary a specific transcription factor, Pit-1, is present; this is essential for the expression of TRβ2 mRNA, and it interacts, among others, with CBP (23).

In view of our recent study on IL-12$^{-/-}$ mice and the results of the IL-18$^{-/-}$ mice, an alternative explanation could be that the lack of IL-18 or IL-12 resulted in the absence of a dominant cytokine that is essential for decreasing D1 activity in the pituitary. Both IL-12 and IL-18 induce IFNγ, which could interfere with many events in thyroid hormone metabolism, especially in the thyroid (3). It is known that LPS administration results in increased IL-12 and IL-18 expression, which subsequently synergistically enhances INFγ mRNA transcription by activating Stat4 and AP-1. The enhanced IFNγ mRNA expression might be responsible for decreased D1 activity in the pituitary after LPS administration, as confirmed by *in vitro* studies (14). Therefore, we also studied the effects of LPS on the pituitary of IFNγR$^{-/-}$ mice compared to their WT. However, LPS administration did not decrease pituitary D1 activity in either IFNγR$^{-/-}$ or WT mice; in contrast, an increase in D1 activity was observed in WT mice. This could be due to the genetic background of these mice (129/Sv/Ev), which is different from IL-12$^{-/-}$ (Balb/c) and IL-18$^{-/-}$ (C57/Bl6) mice. Subtle differences in tissue-specific factors might prevent LPS-induced effects in the pituitary, although the absence of IFNγ signalling results in higher pituitary D1 activity (absolute values) during 24 h compared to WT mice.

In conclusion, LPS administration induced in IL-18$^{-/-}$, IFNγR$^{-/-}$ and WT mice a sustained fall in liver D1 mRNA accompanied by a decrease in serum T_3 and T_4, indicating that neither IL-18 nor IFNγ alone is essential in LPS-induced alterations of peripheral thyroid hormone metabolism. However, the LPS-induced decrease of D1 activity in the pituitary is absent in IL-18$^{-/-}$ mice. The data suggest that IL-18 is involved in the regulation of the central part of the HPT axis during illness, although we cannot rule out the influence of other IL-18-related factors. Whether local production of cytokines by folliculostellate cells in the pituitary (24) or by Kupffer cells in the liver (25) contributes to tissue-specific differences in responses of deiodinase expression to LPS stimulation in these knockout mouse models remains to be studied.

Acknowledgements

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