

Differential effects of leptin and refeeding on the fasting-induced decrease of pituitary type 2 deiodinase and thyroid hormone receptor β 2 mRNA expression in mice

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Abstract

Profound changes in thyroid hormone metabolism occur in the central part of the hypothalamus–pituitary–thyroid (HPT) axis during fasting. Hypothalamic changes are partly reversed by leptin administration, which decreases during fasting. It is unknown to what extent leptin affects the HPT axis at the level of the pituitary. We, therefore, studied fasting-induced alterations in pituitary thyroid hormone metabolism, as well as effects of leptin administration on these changes. Because refeeding rapidly increased serum leptin, the same parameters were studied after fasting followed by refeeding. Fasting for 24 h decreased serum T_3 and T_4 and pituitary TSH β , type 2 deiodinase (D2), and thyroid hormone receptor β 2 (TR β 2) mRNA expression. The decrease in D2 and TR β 2 mRNA expression was prevented when 20 μ g leptin was administered twice during fasting. By contrast, the decrease in TSH β

mRNA expression was unaffected. A single dose of leptin given after 24 h fasting did not affect decreased TSH β , D2, and TR β 2 mRNA expression, while 4 h refeeding resulted in pituitary D2 and TR β 2 mRNA expression as observed in control mice. Serum leptin, T_3 , and T_4 after refeeding were similar compared with leptin administration. We conclude that fasting decreases pituitary TSH β , D2, and TR β 2 mRNA expression, which (with the exception of TSH β) can be prevented by leptin administration during fasting. Following 24 h fasting, 4 h refeeding completely restores pituitary D2 and TR β 2 mRNA expression, while a single leptin dose is ineffective. This indicates that other post-ingestion signals may be necessary to modulate rapidly the fasting-induced decrease in pituitary D2 and TR β 2 mRNA expression.

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Introduction

During fasting, profound changes occur in the hypothalamic part of the hypothalamic–pituitary–thyroid axis (HPT axis), i.e. increased type 2 deiodinase (D2) activity in the arcuate nucleus (ARC) (Diano *et al.* 1998) and decreased thyrotropin-releasing hormone (TRH) expression in the paraventricular nucleus (PVN) (Blake *et al.* 1991). Locally produced T_3 by D2 has been proposed to contribute to the decrease in TRH expression in the PVN, which induces persistently decreased serum thyroid hormones via decreased pituitary thyroid-stimulating hormone (TSH) subunit mRNA expression (Wiersinga 2005).

The observed modest increase in hypothalamic D2 activity during fasting (Diano *et al.* 1998) is in contrast with the rapid, twofold increase in D2 activity (Fekete *et al.* 2004) and mRNA expression in the hypothalamus (Boelen *et al.* 2004) during acute inflammation as observed in an animal model of illness. It has been hypothesized that cytokines might be involved in the inflammation-related changes in hypothalamic D2 expression, while other factors might be involved in fasting-related changes. It is known that

corticosteroids (van Haasteren *et al.* 1995) and leptin are involved in the fasting-induced decrease of TRH expression in the PVN. TRH mRNA expression is downregulated in the PVN during fasting and partly restored by leptin administration (Legradi *et al.* 1997). Furthermore, leptin administration during fasting reverses the increase in serum corticosterone in rats (Ahima *et al.* 1996) and a recent study by Coppola *et al.* (2005) has shown that only a combination of lowered serum leptin levels and increased serum glucocorticoid concentrations resulted in elevated hypothalamic D2 activity during fasting.

Leptin, an adipocyte-derived hormone, regulates food intake, energy expenditure, and endocrine function by signaling via specific hypothalamic pathways. It affects TRH synthesis both directly and indirectly via the TRH gene promoter (Guo *et al.* 2004) and via pro-opiomelanocortin (POMC) and agouti-related protein (AGRP) expression in the ARC (Flier *et al.* 2000) respectively. It is, however, unknown how leptin influences pituitary thyroid hormone metabolism during fasting. The anterior pituitary expresses

both leptin and leptin receptors in TSH-producing cells (Jin *et al.* 2000). Furthermore, incubation of rat pituitary cells with leptin results in an increase of TSH β mRNA expression (Chowdhury *et al.* 2004), although others have shown that isolated hemi-pituitaries of the rat respond to leptin by decreasing TSH release (Ortiga-Carvalho *et al.* 2002).

In summary, food deprivation induces downregulation of the HPT axis at various levels, while serum leptin decreases as well. Refeeding restores fasting-induced HPT axis changes, while exogenous leptin administration restores these changes only to some extent. We, therefore, hypothesized differential effects of refeeding and leptin administration to fasted mice on deiodinase, TSH, and thyroid hormone receptor (TR) gene expression in the anterior pituitary. The aim of the present study was, therefore, to evaluate fasting-induced alterations in pituitary TSH β , D2, and TR β 2 mRNA expression, as well as the effect of leptin administration on these changes. Because refeeding results in a rapid increase in leptin levels, the expression of TSH β , D2, and TR β 2 mRNA was also studied upon refeeding after fasting.

Materials and Methods

Animals

Female Balb/c mice (Harlan Sprague Dawley, Horst, The Netherlands) weighing approximately 20 g were used at 6–12 weeks of age. The mice were kept in 12 h light:12 h darkness cycles (lights on from 0700 to 1900 h), in a temperature-controlled room (22 °C) and food and water were available *ad libitum*. A week before and during the experiment, the mice were housed in groups according to the experimental setup. We performed three types of experiments and the study was approved by the Local Animal Welfare Committee of the Academic Medical Center, University of Amsterdam.

Experimental protocols

Experiment 1 Two groups of mice ($n=6$ /group) were fasted for 24 h, one group received 20 μ g leptin/0.5 ml 0.01 M NaHCO₃ intraperitoneally (recombinant mouse leptin, obtained from Dr Parlow, Harbor-UCLA Medical Center, Torrance, CA, USA). Leptin was administered twice (at 0900 h ($t=0$ h) and 1700 h ($t=8$ h)) in this period. The other group received vehicle. Both groups were compared with control mice ($n=6$), that received vehicle and had free access to food.

Experiment 2 Two groups of mice ($n=6$ /group) were fasted for 24 h and, subsequently, leptin was given at 0900 h ($t=24$ h, i.p. 20 μ g/0.5 ml 0.01 M NaHCO₃). One group was killed at 28 h and the other at 32 h. Both groups were compared with fed control mice that also received leptin at 24 h and were killed at 28 and 32 h.

Experiment 3 Two groups of mice ($n=6$ /group) were fasted for 24 h and refed for either 4 or 24 h. Food was reintroduced at 0900 h. The groups were compared with fed control mice, which were also euthanized at the same time points.

Because of the diurnal variation of thyroid hormone-related genes, the experiments were performed using the same time schedule starting at 0900 h ($t=0$). Furthermore, each time point has its own control group. In all experiments, mice were anesthetized with isoflurane, blood was taken by cardiac puncture and then mice were killed by cervical dislocation. Serum was stored at -20 °C until analyzed. The pituitary and hypothalamus (defined rostrally by the optic chiasma, caudally by the mamillary bodies, laterally by the optic tract, and dorsally by the apex of the third ventricle) were isolated and stored immediately in liquid nitrogen. Based on anatomical landmarks (the apex of the third ventricle (Franklin 1997)), the PVN was obtained by punching the hypothalamus with a hollow needle (diameter 1100 μ m).

Serum determinations

Serum T₃ and T₄ were measured with in-house RIAs (Wiersinga & Chopra 1982). Serum leptin levels were measured with ELISA (Crystal Chem, Inc., Downers Grove, IL, USA). To prevent interassay variation, all samples of one experiment were measured within the same assay.

RNA isolation and RT-PCR

mRNA was isolated from the PVN and pituitary using the Magna Pure apparatus and the Magna Pure LC mRNA isolation kit II (tissue; Roche Biochemicals) according to the manufacturer's protocol and cDNA synthesis was performed with the first strand cDNA synthesis kit for reverse transcriptase (RT)-PCR (AMV; Roche Molecular Biochemicals). Published primer pairs were used to amplify hypoxanthine phosphoribosyl transferase (HPRT), a house-keeping gene (Sweet *et al.* 2001). We designed primer pairs for D2, TR β 2, TSH β , and preproTRH (Boelen *et al.* 2004). Real-time PCR was performed for quantitation of the above-mentioned mRNAs. cDNA standards for the different mRNAs were prepared from RNA of murine liver, pituitary, or hypothalamus. For each mRNA assayed, a standard curve was generated using tenfold serial dilutions of this target standard PCR product and the same primers used to amplify the cDNA. For each gene, the standard protocol was optimized by varying MgCl₂ concentrations. PCR were setup with cDNA, MgCl₂ (25 mM), SybrGreenI (Roche Molecular Biochemicals), forward and reverse primer and H₂O. The reactions were then cycled in the LightCycler (Roche Molecular Biochemicals) as described before (Boelen *et al.* 2004). The LightCycler software generated a standard curve (measurements taken during the exponential phase of

the amplification), which enabled the amount of each mRNA in each test sample to be determined. All results were corrected as to their mRNA content using HPRT mRNA. Samples were individually checked for their PCR efficiency (Ramakers *et al.* 2003). The median of the efficiency was calculated for each assay and samples with a greater than 0.05 difference of the efficiency median value were not taken into account.

Statistical analysis

Data were normally distributed (Shapiro–Wilk test) and are presented as the mean \pm s.e.m. Variation between fasted mice, treated with leptin or refed and control fed mice, was evaluated by ANOVA with two grouping factors (time and treatment) and their interaction followed by Tukey's test for pair-wise comparisons (*P* values in the figures represent the pair-wise *P* values). In case of unequal variances, data were rank transformed prior to the ANOVA. All analyses were carried out in SPSS 11.5.1 (SPSS, Inc., Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

Results

Fasting-induced hypothalamic preproTRH mRNA expression with and without leptin administration

We measured preproTRH mRNA expression by RT-PCR in the PVN in order to check our experimental setup. PreproTRH mRNA expression decreased after 24 h fasting and this decrease was prevented by leptin given twice during the fasting period (Fig. 1A). ANOVA analysis showed that leptin administration after 24 h to fasted animals resulted in significant preproTRH mRNA expression compared with

fed animals which was caused by the fasting-induced decrease at 24 h. No differences in starved and fed mice were observed at 4 and 8 h after leptin administration (Fig. 1B). We, therefore, conclude that we have administered adequate amounts of leptin to study differential effects of leptin and refeeding on pituitary thyroid hormone metabolism selectively.

Effect of leptin administration during fasting on thyroid hormone metabolism (Experiment 1)

Fasting resulted in significantly decreased serum leptin and thyroid hormone levels. Leptin administration, given twice during the fasting period, rapidly increased serum leptin levels although the half-life of mouse recombinant leptin is too short ($t_{1/2}$ = 3 h (Ahima *et al.* 1996)) to maintain high serum leptin levels during 24 h fasting similar to control mice. Serum T₃ and T₄ levels were slightly higher in the leptin-treated group compared with starved mice, but still significantly lower compared with fed control mice (Table 1). Fasting also resulted in decreased pituitary TSH β , D2, and TR β 2 mRNA expression compared with fed control mice (Fig. 2). The decrease in TSH β mRNA expression was not prevented by leptin administration, in contrast to the fasting-induced decrease of pituitary D2 mRNA expression. The fasting-induced decrease in TR β 2 tends to be prevented by leptin administration (*P* = 0.09; Fig. 3).

Effect of leptin administration after fasting on thyroid hormone metabolism (Experiment 2)

Leptin administration after 24 h fasting resulted in significantly higher leptin levels at 28 h and lower levels at 32 h, which is in agreement with the half-life of the hormone ($t_{1/2}$ = 3 h). ANOVA analysis showed a significant difference

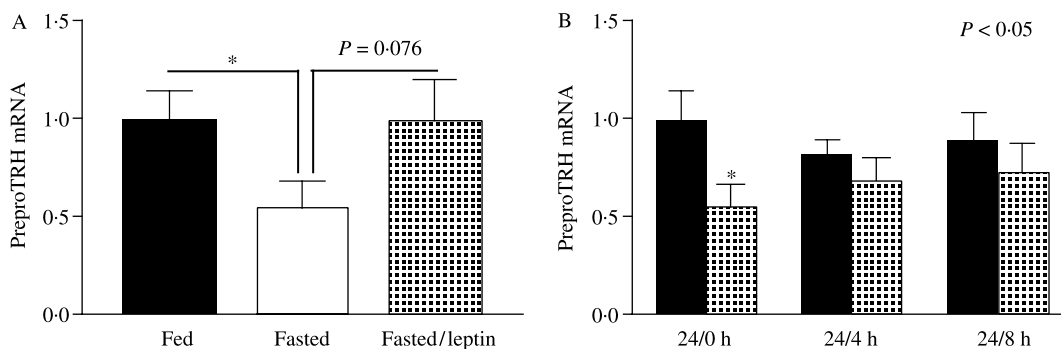


Figure 1 (A) Experiment 1: relative expression (normalized with the mean value of the 24 h control group, which was set at 1) of preproTRH mRNA in the paraventricular nucleus (PVN) of mice after 24 h fasting, while leptin was administered twice (at 0 and 8 h, 20 μ g/0.5 ml, hatched bars) compared with fasted mice (open bars) and mice which received food *ad libitum* (black bars). (B) Experiment 2: relative expression (normalized to the mean value of the 24 h control group, which was set at 1) of preproTRH mRNA in the PVN of mice after 24 h fasting followed by leptin given once at 24 h euthanized (20 μ g/0.5 ml, hatched bars) compared with mice which received food *ad libitum* (black bars). Mean values (n = 6) \pm s.e.m. of expression relative to hypoxanthine phosphoribosyl transferase (HPRT) are depicted; *P* value indicates treatment difference between groups by ANOVA. Differences between groups at a single time point are indicated by **P* < 0.05.

Table 1 Serum thyroid hormone and leptin levels in mice after 24 h starvation. Leptin was administered twice (at 0 and 8 h, 20 µg/0.5 ml, Experiment 1), after 24 h starvation followed by leptin given once at 24 h (20 µg/0.5 ml, † at 28 and 32 h, Experiment 2) and after 24 h starvation followed by a period of refeeding of 4 or 24 h (‡ at 28 and 48 h, Experiment 3). Each group was compared to its own control group as given in the table. Values were given as mean ± s.e.m.

| | Time (h) | T ₃ (nmol/l) | T ₄ (nmol/l) | Leptin (pg/ml) |
|--------------------------------------------------|----------|-------------------------|-------------------------|----------------|
| Treatment | | | | |
| <i>Experiment 1: leptin given during fasting</i> | | | | |
| Control (food <i>ad libitum</i>) | 24 | 1.15 ± 0.04 | 56 ± 4 | 3303 ± 466 |
| Fasting | 24 | 0.56 ± 0.06* | 25 ± 2* | 285 ± 24* |
| Fasting/leptin | 24 | 0.74 ± 0.03*† | 32 ± 3*† | 447 ± 127* |
| <i>Experiment 2: leptin given after fasting</i> | | | | |
| Control (food <i>ad libitum</i>)/leptin | 24/4 | 1.13 ± 0.11 | 61 ± 7 | 2904 ± 436 |
| Fasting/leptin | 24/4 | 0.51 ± 0.10* | 24 ± 3* | 4902 ± 1293 |
| Control (food <i>ad libitum</i>)/leptin | 24/8 | 0.93 ± 0.03 | 47 ± 6 | 2418 ± 541 |
| Fasting/leptin | 24/8 | 0.58 ± 0.10* | 20 ± 2* | 743 ± 263* |
| <i>Experiment 3: refeeding after fasting</i> | | | | |
| Control (food <i>ad libitum</i>) | 24 | 1.18 ± 0.10 | 69 ± 7 | 2330 ± 327 |
| Fasting | 24 | 0.75 ± 0.11* | 42 ± 5* | 182 ± 32* |
| Control (food <i>ad libitum</i>) | 28 | 1.17 ± 0.04 | 72 ± 6 | 1889 ± 271 |
| Fasting/refeeding | 24/4 | 0.84 ± 0.03* | 41 ± 3* | 2402 ± 433 |
| Control (food <i>ad libitum</i>) | 48 | 1.20 ± 0.06 | 74 ± 6 | 1989 ± 297 |
| Fasting/refeeding | 24/24 | 0.88 ± 0.11‡ | 56 ± 4‡ | 3630 ± 242* |

* $P < 0.01$ and † $P < 0.05$ compared with control treatment. ‡ $P < 0.05$ compared with fasted mice.

in serum T₃ ($P < 0.001$) and T₄ levels ($P < 0.001$) between fasted mice, which received a single dose of leptin compared with fed mice which received leptin. The fasting-induced decrease in serum T₃ and T₄ was not restored after leptin administration and the levels were similar to the concentrations observed after 24 h fasting (Table 1). The fasting-induced decrease of TSHβ mRNA expression was also not restored 4 and 8 h after leptin injection compared with fed mice which received leptin (ANOVA, $P < 0.001$; Fig. 4A). ANOVA analysis revealed a significant difference in pituitary D2 ($P < 0.05$) and TRβ2 ($P < 0.01$) mRNA expression between leptin given after 24 h fasting and to fed mice. This difference was restricted to 4 h after injection. Eight hours after leptin administration, pituitary D2 and TRβ2 mRNA expression was not different compared with control mice

(Fig. 4B and C). Altered TRβ2 and TSHβ mRNA expression in the fed control groups throughout the day reflects the circadian rhythm of these genes (TRβ2, $P_{\text{time}} < 0.01$ and TSHβ, $P_{\text{time}} < 0.05$).

Effect of refeeding on fasting-induced changes in thyroid hormone metabolism (Experiment 3)

Refeeding resulted in markedly increased serum leptin levels after 4 and 24 h, which were not different (ANOVA, not significant) compared with fed control mice (although *post hoc* analysis indicates a significant difference at 24 h). Refeeding, however, did not result in a complete recovery of serum T₃ (ANOVA, $P < 0.001$) and T₄ (ANOVA, $P < 0.001$) levels after 4 h and even after 24 h of refeeding compared with control mice

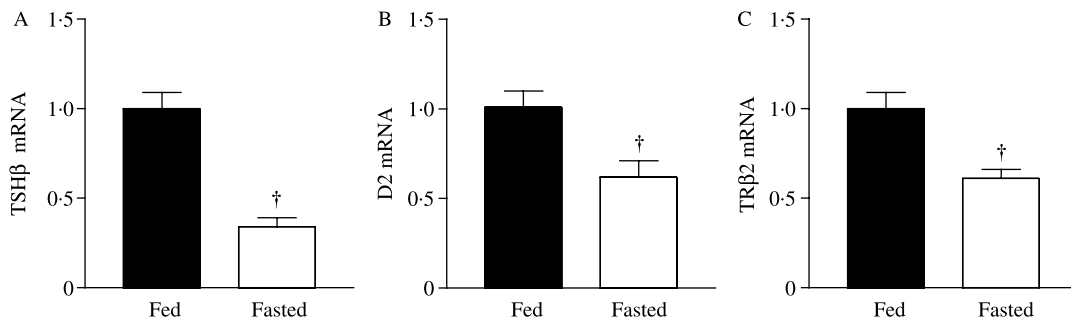


Figure 2 Relative expression (normalized with the mean value of the 24 h control group which was set at 1) of (A) thyroid-stimulating hormone β (TSHβ), (B) type 2 deiodinase (D2), and (C) thyroid hormone receptor β2 (TRβ2) mRNA in the pituitary of mice after 24 h fasting (open bars) compared with control mice which received food *ad libitum* (black bars). Mean values ($n = 12$) ± s.e.m. of expression relative to HPRT are depicted; differences between groups at a single time point are indicated by † $P < 0.01$.

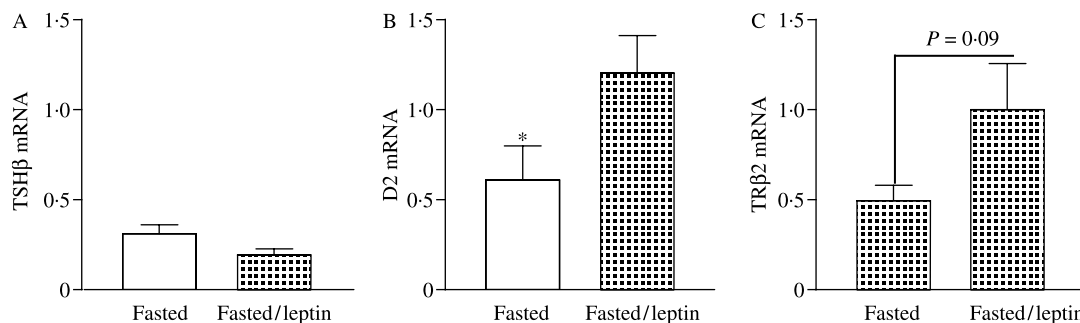


Figure 3 Relative expression (normalized with the mean value of the 24 h control group which was set at 1) of (A) TSH β , (B) D2, and (C) TR β 2 mRNA in the pituitary of mice after 24 h fasting, while leptin was administered twice (at 0 and 8 h, 20 μ g/0.5 ml, hatched bars) compared with fasted mice (open bars). Mean values ($n=6$) \pm s.e.m. of expression relative to HPRT are depicted; differences between groups at a single time point are indicated by * $P < 0.05$.

(Table 1). Refeeding resulted in similar pituitary D2 and TR β 2 mRNA expression compared with fed control mice at 4 and 24 h, while pituitary TSH β mRNA expression was statistically different after refeeding compared with fed control mice ($P < 0.01$). *Post hoc* analysis indicated a significant difference at 0 and 4 h refeeding, but not after 24 h refeeding (Fig. 5).

Discussion

The aim of the present study was to evaluate (1) fasting-induced changes in pituitary TSH β , D2, and TR β mRNA expression of mice and (2) differential effects of leptin administration and refeeding on these changes. It is known that leptin administered during fasting reverses the fasting-induced decrease in TRH expression in the PVN (Legradi *et al.* 1997). We therefore punched the PVN area of the hypothalamus and measured preproTRH mRNA expression in order to validate our model, i.e. whether we used adequate amounts of leptin to influence the hypothalamic part of the HPT axis. A decrease in preproTRH mRNA expression was observed after 24 h fasting and this decrease was

prevented by our leptin schedule. We, therefore, concluded that we administered adequate amounts of leptin to study the role of leptin on pituitary thyroid hormone metabolism.

Fasting induced decreased pituitary TSH β , D2, and TR β mRNA expression as well as decreased serum thyroid hormone levels. We studied the influence of leptin administration given twice during the fasting period, a single dose of leptin given after the fasting period, and endogenous leptin induced by refeeding on the fasting induced alterations.

Leptin administration during fasting did not prevent the decrease in TSH β mRNA expression despite restored hypothalamic preproTRH mRNA expression. In contrast, pituitary D2 and TR β mRNA expression normalized when leptin was administered during the fasting period. The fasting-induced decrease in pituitary TSH β mRNA expression was also not restored within 8 h after leptin administration. Our results seem in contrast with a previous study reporting that leptin intracerebrovascularily administered after a period of 48 h fasting restored the fasting-induced decrease in serum TSH in rats (Seoane *et al.* 2000), which might be due to the different route of administration (i.c.v. vs i.p.). Our results also, did not agree completely with the study of Ortiga-Carvalho

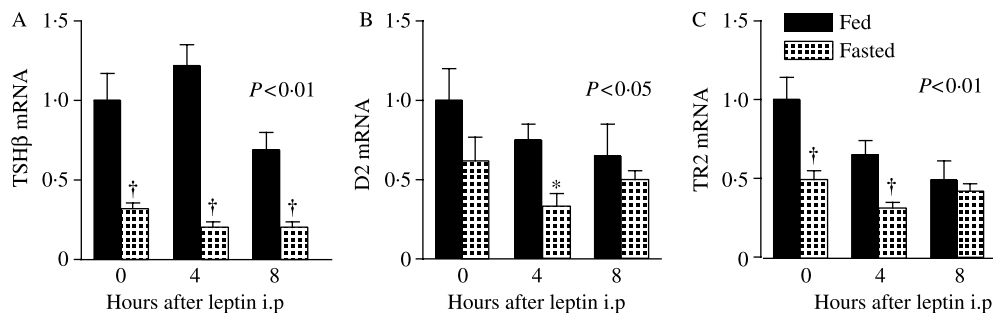


Figure 4 Experiment 2: relative expression (normalized to the mean value of the 24 h control group which was set at 1) of (A) TSH β , (B) D2, and (C) TR β 2 mRNA in the pituitary of mice after 24 h fasting followed by leptin given once at 24 h (20 μ g/0.5 ml, euthanized after 4 and 8 h) compared with mice which received food *ad libitum* and leptin (20 μ g/0.5 ml, † after 4 and 8 h). Mean values ($n=6$) \pm s.e.m. of expression relative to HPRT are depicted; P value indicates treatment difference between groups by ANOVA. Differences between groups at a single time point is indicated by * $P < 0.05$ and † $P < 0.01$.

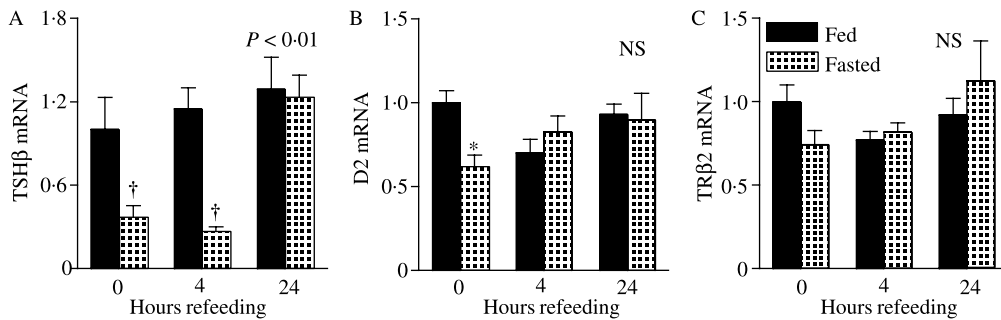


Figure 5 Experiment 3: relative expression (normalized to the mean value of the 24 h control group which was set at 1) of (A) TSH β , (B) D2, and (C) TR β 2 mRNA in the pituitary of mice after 24 h fasting followed by a period of refeeding (4 h, euthanized at 28 and 24 h) compared with mice which received food *ad libitum*. Mean values ($n=6$) \pm S.E.M. of expression relative to HPRT are depicted; P value indicates treatment difference between groups by ANOVA. Differences between groups at a single time point are indicated by * $P < 0.05$ and † $P < 0.01$, NS: not significant.

et al. (2002), who showed that leptin administration to fed rats resulted in an increase in serum TSH after 2 h, although the leptin-treated group was not compared with a control group at the same time point, so an effect of the diurnal rhythm cannot be excluded. Furthermore, they administered 0.08 and 0.16 μg leptin/g body weight (BW) (in our study, mice received 1 μg /g BW) and only administration of 0.08 μg leptin/g BW increased serum TSH levels, while administration of 0.16 μg leptin/g BW did not have an effect, which is in agreement with our results (Ortiga-Carvalho *et al.* 2002). However, notwithstanding the fact that we cannot exclude the possibility that pituitary TSH β mRNA expression was not restored 8 h after leptin injection, because of lower serum leptin levels compared with those in fed mice, different mechanisms might be involved in the fasting-induced decrease in pituitary TSH β vs D2 and TR β 2 mRNA expression. Restored pituitary D2 and TR β 2 mRNA expression might be leptin and/or TRH dependent, while the fasting-induced decrease in pituitary TSH β appears to be not. The cellular expression of these genes are not likely to explain the observed differences because Li & Boyages (1997) demonstrated specific (nuclear) localization of TR β 2 protein in TSH-producing cells of the anterior pituitary in the rat, whereas Alkemade *et al.* (2005) showed that in human pituitary D2 does not co-localize with TSH, but is expressed in pituitary folliculate-stellate (FS) cells. It has been postulated that leptin has a predominant role in the fasting-induced changes in pituitary TSH β expression via decreased TRH expression in the PVN (Seoane *et al.* 2000), but our results do not support this hypothesis. Many other hypothalamic neuropeptides (i.e. POMC, neuropeptide Y (NPY), AGRP, growth hormone-releasing hormone, and opioids) and humoral factors are affected by fasting (Shi *et al.* 1993, Swart *et al.* 2002), and, perhaps, these factors are involved in pituitary TSH β mRNA expression during fasting independently of leptin. Furthermore, specific proteins (e.g. Pit-1 and retinoid X receptor- γ) expressed amongst others in the thyrotropic cells play an important role in TSH β gene expression (Shupnik 2000), and it is possible that a period of fasting affects these proteins also independently of leptin. A

recent study has shown that neuromedin B (NB), a bombesin-related peptide, highly concentrated in the pituitary and expressed in the thyrotropic cell (Steel *et al.* 1988) is involved in TSH gene regulation (Oliveira *et al.* 2006). It has been postulated that NB is an inhibitor of TSH secretion, while it is also known that NB increased during fasting and therefore is probably responsible for the decrease in TSH secretion (Ortiga-Carvalho *et al.* 1997). The *in vivo* effects of leptin on NB are not very consistent, only very low doses given to fed rats decreased NB within 2 h, while higher doses of leptin did not have an effect (Ortiga-Carvalho *et al.* 2003) possibly explaining why the fasting-induced decrease in pituitary TSH β mRNA cannot be restored by leptin administration in our experimental setting.

Leptin exerts its action via the leptin receptor (OB-R), which belongs to the family of class I cytokine receptors and is present in many tissues, including normal mouse pituitary and a mouse FS cell line (Jin *et al.* 2000). Activation of this receptor results in the activation of the cytokine-related JAK/STAT signaling pathway. Leptin is also capable of activating nuclear factor (NF)- κ B in dendritic cells (Mattioli *et al.* 2005) suggesting that leptin might activate NF- κ B in pituitary FS cells, as it is known that these cells use the NF- κ B signaling pathway (Lohrer *et al.* 2000).

Fekete *et al.* (2004) have shown that the D2 gene can be activated by the NF- κ B second messenger system in an *in vitro* cell system and, therefore, the activation of NF- κ B in FS cells via leptin might be an explanation for the normalization of pituitary D2 mRNA expression. The experimental design (time-course, moment, and amount of leptin given), however, seems critical because a single injection of leptin after 24 h fasting is not enough to restore pituitary D2 expression within 4 h, while leptin given during the fasting period completely prevents the fasting-induced decrease in pituitary D2 mRNA expression. The alterations in pituitary TR β 2 mRNA expression correlate positively with those in pituitary D2 mRNA expression. D2 gene expression, however, is negatively regulated at the transcriptional level by T $_3$, probably via the TR β (Gereben & Salvatore 2005),

suggesting that decreased TR β 2 mRNA does not explain decreased pituitary D2 mRNA expression.

Fasting induces decreased TR β 2 mRNA expression and leptin prevents this decrease only when administered during fasting. It is known that serum-free fatty acid (FFA) concentrations increase during fasting (Suzuki *et al.* 2002). Effects of FFA on thyroid hormone receptor function have been known for many years, especially inhibition of thyroid hormone binding to the TR (Wiersinga *et al.* 1988). It has also been shown that sodium butyrate, a short-chain fatty acid, inhibits TR β 2 mRNA expression selectively by rapidly repressing TR β 2 gene transcription (Lazar 1990). Leptin administration decreases serum FFA concentrations (Kalaivanisailaja *et al.* 2003), which thus could partly explain restored pituitary TR β 2 mRNA expression as observed in the present study. Peroxisome proliferator activated receptor- γ , another nuclear receptor of the thyroid hormone family, decreases significantly in the pituitary after 24 h fasting, probably via FFA (Nakamura *et al.* 2004) without changes in the basal hypothalamus, which supports this notion (Wiesner *et al.* 2004).

The observed alterations in pituitary thyroid hormone metabolism were compared with those in starved animals who were refed in order to differentiate between effects of leptin *per se* and refeeding. Although serum leptin reached similar levels after 4 h refeeding and leptin treatment, altered pituitary D2 and TR β 2 mRNA expression was completely abolished within 4 h by refeeding, but not by leptin administration. This suggests that other post ingestion signals in addition to leptin are necessary to influence pituitary D2 and TR β 2 mRNA expression rapidly. Eight hours after leptin treatment, however, pituitary D2 and TR β 2 mRNA expression were not different compared with control mice indicating that leptin alone influences pituitary D2 and TR β 2 mRNA expression more slowly. This might be explained by the different response of corticosterone on leptin administration or refeeding. Ahima *et al.* (1996) showed that the fasting-induced increase in serum corticosterone was partly prevented by leptin administration, while it has been shown that refeeding for a period of 60 min resulted in complete recovery of serum corticosterone levels induced by food-deprivation in rats (Millward *et al.* 1983). Nevertheless, our results are in agreement with a study of Swart *et al.* (2002) reporting that refeeding partly restores fasting-induced alterations in hypothalamic NPY and POMC mRNA expression within 6 h, while leptin administration after food deprivation is not sufficient to affect mRNA levels.

In conclusion, fasting decreases pituitary TSH β , D2, and TR β 2 mRNA expression and leptin treatment during the fasting period prevents the decrease in D2 and TR β 2 mRNA, but not in TSH β mRNA expression. Leptin administration after fasting influences D2 and TR β 2 mRNA expression only after 8 h, while refeeding (which also results in high serum leptin levels) restores D2 and TR β 2 mRNA expression completely within 4 h. Leptin may, theoretically, influence these genes by several mechanisms (decreasing FFA

concentrations, activating the NF κ B pathway), but our results indicate that other post ingestion signals in addition to leptin may be necessary to rapidly modulate the fasting-induced decrease in pituitary D2 and TR β 2 mRNA expression.

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