Changes in hepatic insulin-like growth factor-binding proteins -1, -2 and -3 mRNA levels in rats with altered thyroid status

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Abstract
Changes in thyroid status have a major effect on the GH/insulin-like growth factor (IGF) axis. The majority of IGF in the circulation is bound to specific IGF-binding proteins (IGFBPs) of which six have been cloned and sequenced. We have studied changes in hepatic gene expression of IGFBP-1, -2 and -3, in male Wistar rats rendered hyperthyroid (thyroxine, 200 µg/kg per day) or hypothyroid (propylthiouracil, 0-1% daily). Littermates of the same age were used as controls (n=6 in each group). Thyroxine was measured by radioimmunoassay, and hepatic IGFBP-1, -2 and -3 mRNA levels by Northern blot analysis using specific rat cDNA probes with a 28S ribosomal probe as a loading control. Mean ± s.e.m. thyroxine levels were 247±0±44-5 (hyperthyroid group), <9-0 (hypothyroid group) and 76-0±4±5 nmol/l (control group). IGFBP-1 and -2 mRNA levels in the hypothyroid animals compared with the controls were significantly increased, but similar levels of expression were found in thyrotoxic and control rats. IGFBP-3 mRNA levels in hypothyroid animals were decreased, and increased in thyrotoxic animals. Thus, in the adult rat, hypothyroidism is associated with increased hepatic IGFBP-1 and -2 gene expression, but decreased IGFBP-3 gene expression, while in thyrotoxic animals there are normal IGFBP-1 and -2 mRNA levels but increased IGFBP-3 gene expression. These results suggest that there is specific and different transcriptional regulation for IGFBP-1, -2 and -3 in hypo- and hyperthyroid rats.

Journal of Endocrinology (1994) ¹⁴⁰, 251–255

Introduction
The majority of insulin-like growth factor (IGF)-I and -II circulate bound to specific high-affinity binding proteins (IGFBPs) (Zapf et al. 1975, Hintz & Liu 1977), of which six, IGFBP-1 to -6, have been isolated and characterized to date (Shimasaki & Ling 1991). These binding proteins modulate the function of IGFs, forming part of a highly sophisticated regulatory system (Rosenfeld et al. 1991). At present, we only have a detailed understanding of the physiology of IGFBP-1 and -3, although the availability of specific radioimmunoassays is rapidly increasing our knowledge of the pathophysiology of IGFBP-2.

IGFBP-1 is a small molecular weight protein (25 kDa) whose serum levels fluctuate widely over a 24-h period, and are inversely correlated with those of insulin (Baxter & Cowell 1987, Suikkari et al. 1988). IGFBP-1 levels are increased by trauma, pregnancy, diabetes and starvation. In the majority of bioassays systems, IGFBP-1 acts as an inhibitor of anabolic IGF actions (Ritvos et al. 1988, Taylor et al. 1990). Preliminary data suggest that IGFBP-1 production from Hep G2 cells in culture is increased in vitro by the addition of tri-iodothyronine (Angervo et al. 1993), and hyperthyroidism in man is associated with an increase in IGFBP-1 levels (Miell et al. 1993).

IGFBP-2 levels are increased during starvation and diabetes in the rat. In man, high levels are found in hypopituitary patients and following fasting (Clemmons et al. 1991). IGFBP-2 levels in man are unaltered by the administration of thyroxine to normal volunteers (Blum et al. 1993) but preliminary data suggest an increase during hypothyroidism (J Miell, unpublished observation). In the rat, congenital hypothyroidism is associated with an increase and prolongation of hepatic IGFBP-2 expression (Nanto-Salonen et al. 1991). Otherwise very little is known of the function and regulation of IGFBP-2.

IGFBP-3 is a 53 kDa protein that in normal physiological states associates with an acid-labile subunit and with IGF-I or -II to form a 150 kDa complex (Baxter & Martin 1989). Factors regulating IGFBP-3 include age, nutritional status, growth hormone (GH) and IGF-1 levels. IGFBP-3 itself may modulate the functions of IGFs at a cellular level. In bioassay systems, the addition of IGFBP-3 may potentiate or inhibit the biological actions of IGFs (De Mellow...
& Baxter 1988). In hypothyroidism in man, IGFBP-3 levels have been reported as low, rising towards normal on treatment (Mielj et al. 1993).

We have previously reported changes in circulating IGF-1 and hepatic IGF-1 gene expression in rats with altered thyroid status (Thomas et al. 1993). To further our understanding of the relationship between thyroid hormones and IGFs, we have studied changes in the levels of IGFBP-1, -2 and -3 mRNA in the liver of adult male Wistar rats rendered hypothyroid and hyperthyroid.

Materials and Methods

Male Wistar Rats (Bantin and Kingman, Hull, N. Humberside, UK) were caged individually in a temperature-controlled room (22 ± 1 °C), with a 12-h light:darkness cycle. Six-week-old rats were divided into three age-matched groups (n=6 in each group). They were rendered hypothyroid or hypothyroid by the addition to the drinking water of, respectively, 0·1% propylthiouracil and 200 µg thyroxine/kg per day for 12 weeks (Rohner & Dillman 1988). Littermates of the same age were used as controls. All groups had unrestricted access to laboratory rodent food (Quest Nutrition, Canterbury, Kent, UK) and water during the study.

At the end of the 12-week period, the rats were killed, and the livers aseptically removed, snap-frozen in liquid nitrogen, and kept at −70 °C until analysis. Mixed arterial-venous trunk blood was taken, spun and the serum stored at −20 °C until analysis.

Serum thyroxine

Serum thyroxine was measured using a commercial radioimmunoassay kit (Diagnostic Products Ltd, Abingdon, Oxon, UK). The detection limit of this assay was 9·0 nmol/l.

RNA Northern analysis

Specific mRNA steady-state levels were determined in total RNA extracted from the liver using guanidium thiocyanate–phenol–chloroform extraction as described by Chomczynski & Sacchi (1987). Animal specimens were analysed individually. Total RNA (10 µg) from individual animal livers were denatured in guanidyl and dimethylsulphoxide, and applied to a 1% agarose gel. Ethidium bromide staining of the gels confirmed even loading of the lanes. After electrophoresis, the RNA was transferred onto nylon filters (Hybond N; Amersham International plc, Amersham, Bucks, UK) by capillary action and fixed by alkali fixation. The blots were prehybridized for 4 h at 42 °C in 50% formamide, 2% (w/v) sodium dodecyl sulphate (SDS), 10% dextran sulphate and 200 µg denatured salmon sperm DNA/ml. Hybridization was performed in the same solutions for 20 h at 42 °C. The membranes were hybridized with cDNA probes for IGFBP-1, -2 and -3, and labelled using an oligolabelling kit (Pharmacia, Uppsala, Sweden) according to the manufacturer’s directions, with α-32P dCTP (specific activity 3000 Ci/mmol); (Amersham International plc). After hybridization, the membranes were washed twice at room temperature in 2 × SSC and 0·1% SDS for 30 min, and twice at 65 °C in 0·1% SSC and 0·1% SDS for 30 min (1 × SSC is 0·15 m NaCl, 0·015 m sodium citrate). The membranes were exposed to autoradiography at −70 °C on Kodak X-Omat AR film. To check for equal loading the membranes were stripped and rehybridized with a 28S ribosomal probe.

Quantification of relative mRNA ratios of specific hybridization bands were obtained by laser densitometry scanning of autoradiographs using a laser densitometer (Ultrascan; LKB Instruments, Bromma, Sweden). The results are expressed in arbitrary densitometric units as a ratio of the densitometric values for IGFBP-1, -2 and -3, divided by the ribosomal 28S signal.

Statistical analyses

The results are expressed as mean ± s.e.m. Comparison of the various groups was carried out by analysis of variance, and when P<0·05 statistical analysis was completed using the Wilcoxon rank sum test.

Results

Serum thyroxine was significantly higher in the thyrotoxic group (247±4·5 nmol/l) and below the detection limit of the assay in the hypothyroid group (<9·0 nmol/l) compared with the controls (76±4·5 nmol/l) (P<0·001 vs control group for both hypothyroid and hyperthyroid groups).

Representative Northern blots of hepatic total RNA hybridized to IGFBP-1, -2 and -3 from control, hypothy¬roid and thyrotoxic rats are shown in Fig. 1. IGFBP-1 showed a single transcript (1·7 kb), with greatly increased expression in the hypothyroid animals compared with controls (8·55 ± 2·62 vs 0·53 ± 0·09 arbitrary densitometric units; P<0·003), but there was no difference between the thyrotoxic and control IGFBP-1 mRNA values (0·47 ± 0·20 and 0·53 ± 0·09 respectively). IGFBP-2 showed a single transcript (1·5 kb), scarcely detectable in controls or thyrotoxic rats (0·11 ± 0·04 vs 0·08 ± 0·02), but with an increased expression in the hypothyroid rats (0·71 ± 0·15; P<0·003 vs control). IGFBP-3 showed a single transcript (2·5 kb) with decreased expression in the hypothyroid group (0·07 ± 0·01) compared with the control group (0·27 ± 0·07; P<0·03), and increased expression in the thyrotoxic group (0·63 ± 0·06; P<0·01 vs controls).
**FIGURE 1.** (a) Northern blot analysis of total hepatic RNA from control (lanes 1–3), hypothyroid (lanes 4 and 6) and thyrotoxic rats (lanes 7–9). Lane 5 was empty. The blots were hybridized with IGF-binding protein (IGFBP-1) and IGFBP-2 cDNA probes, and then stripped and rehybridized to a 28S ribosomal RNA probe. (b) Northern blot analysis of total hepatic RNA from control, hypothyroid and thyrotoxic rats. The blot was hybridized with an IGFBP-3 cDNA probe, and then stripped and rehybridized to a 28S ribosomal RNA probe.
Discussion

We have studied the changes in the hepatic gene expression of IGFBP-1, -2 and -3 in rats rendered hyperthyroid or hypothyroid and compared them with control animals. We observed differential regulation of hepatic IGFBP-1, -2 and -3 mRNA levels in response to altered thyroid status. When the animals were hypothyroid there were increased IGFBP-1 and IGFBP-2 and decreased IGFBP-3 mRNA levels, while during thyrotoxicosis there were increased IGFBP-3 but normal IGFBP-1 and IGFBP-2 mRNA levels.

In the rat the IGFBP-1 gene is known to be regulated by a number of factors including age, insulin, GH and glucocorticoids (Ooi et al. 1990). The changes we found in hepatic IGFBP-1 mRNA levels in the adult hypothyroid rat may be a direct effect of thyroid hormones or mediated by a combination of changes in GH, insulin and glucocorticoids, as the mechanism controlling IGFBP-1 mRNA in vivo is complex. We found no changes in IGFBP-1 mRNA in thyrotoxic rats but in man thyrotoxicosis is associated with increased circulating IGFBP-1 levels and decreased IGF bioactivity (Miell et al. 1993).

The factor(s) responsible for the regulation of IGFBP-2 mRNA in the rat are not well defined. Hepatic IGFBP-2 mRNA levels are increased in fasted, diabetic and hypophysectomized rats (Ooi et al. 1990, Orłowski et al. 1990, Tseng et al. 1992). GH treatment of hypophysectomized rats alone, or in combination with thyrxine, testosterone and cortisone acetate does not normalize hepatic IGFBP-2 mRNA (Margot et al. 1989, Ooi et al. 1990), suggesting that other factors may also mediate IGFBP-2 regulation. The increased expression of the IGFBP-2 gene in the liver of our hypothyroid animal model is of considerable interest. Previously, it has been suggested that the effect of thyroid hormones on IGFBPs depends on the developmental stage of the animal, with congenitally hypothyroid rats, but not adult rats, showing a prolonged elevation of serum levels of IGFBP-2 and IGFBP-2 hepatic gene expression (Nanto-Salonen et al. 1991, Nanto-Salonen & Rosenfeld 1992). Our results are in disagreement with Nanto-Salonen & Rosenfeld (1992), as we detected an increase in hepatic IGFBP-2 gene expression in adult hypothyroid rats. This difference in results may be related to prolonged hypothyroidism in our rats (12 weeks) compared with the short duration of their study (2 weeks). The increased expression of IGFBP-2 in hypothyroid rats may be related to their decreased thyroid hormone levels, although, as with IGFBP-1, IGFBP-2 gene regulation appears to be complex and may involve an interplay between different hormones.

In this study, hepatic IGFBP-3 gene expression in hypothyroid rats was found to be decreased, with increased expression in hyperthyroid rats. It has been suggested that IGF-1 regulates IGFBP-3 levels in plasma (Shimasaki & Ling 1991), thus these results may reflect the low IGF-1 levels seen in hypothyroidism and high levels seen in thyrotoxicosis, as in man thyrotoxicosis is associated with increased circulating IGFBP-3 levels (Miell et al. 1993). However, GH is also important in the regulation of IGFBP-3, so the low GH levels seen in hypothyroidism (Bruhn et al. 1992) may contribute to a diminution in the IGFBP-3 hepatic mRNA levels. A direct effect of thyroid hormones on IGFBPs gene expression cannot be excluded as, in different systems in vitro, tri-iodothyronine has been shown to stimulate IGFBP-1, -2 and -3 at a transcriptional level (Ceda et al. 1992, Schmitz et al. 1992, Angervo et al. 1993).

In summary, the results of this study indicate profound differences in the expression of IGFBP-1, -2 and -3 genes with altered thyroid status, and suggest that there is specific transcriptional regulation of the genes that may reflect differences in their biological function. The exact mechanism(s) mediating the differential regulation of IGFBPs, and whether the changes in altered thyroid status are a direct result of changes in thyroid hormones or produced by indirect changes in other metabolic hormones or both, remain to be defined.

Acknowledgements

This work was supported in part by a grant from the Joint Research Board of St Bartholomew’s Hospital. J M is supported by a Wellcome Trust fellowship. The probes were kindly donated by Dr L J Murphy (IGFBP-1) and Dr S Shimasaki (IGFBP-2 and -3). J R-A is supported by grant 93/0747 from Fondo de Investigacion Sanitaria.

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Revised manuscript received 1 June 1993

Accepted 6 September 1993

*Endocrinology* (1994) **140**, 251-255