Effect of thyroxine administration on the IGF/IGF binding protein system in neonatal and adult thyroidectomized rats

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Abstract

The effects of different doses of thyroxine (T₄) delivered by injection or s.c. pellet implantation on alterations of the IGF/IGFBP system were studied in neonatal and adult thyroidectomized (Tx) rats. Body weight, blood glucose, plasma insulin, TSH and GH and pituitary GH content, as well as serum IGF-I, IGF-II, IGFBP-1, -2 and -3 and their liver mRNA expression were assayed. Pellet implantation with the smaller dose of T₄ (1.5 µg/100 g body weight (b.w.) per day) in Tx neonatal rats decreased serum IGF-I, -II and the 30 kDa complex of IGFBPs (IGFBP-1 and -2), and increased serum IGFBP-3. Only the larger dose of T₄ (3 µg/100 g b.w. per day) recovered liver mRNA expression of IGF-I and ensured euthyroid status as shown by the normalized levels of plasma TSH. The rapid increase of body weight and serum GH after T₄ administration indicated a high sensitivity to T₄ during the neonatal period. Serum and liver mRNA expression of IGFs and plasma insulin and GH recovered in adult Tx rats after pellet implantation of 1.75 µg/100 g b.w. per day throughout 10 days. The continuous replacement of T₄ by pellet seems to be the most suitable method for thyroid rehabilitation. A very good correlation was found between insulin and IGF-II in Tx neonates treated with T₄ but not between insulin and IGF-I in Tx adults. IGFBP-2 seems to be up-regulated by T₄ deprivation in neonatal and adult rats. Finally, a good correlation as well as a partial correlation were found between IGFs and thyroid hormones in both neonatal and adult Tx populations, suggesting a direct effect in vivo of T₄ on the hepatic secretion of IGFs, as previously suggested in vitro.

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Introduction

Insulin-like growth factors (IGFs) are peptide hormones with endocrine, paracrine and autocrine modes of action (Jones & Clemmons 1995); and their function is modulated by six types of IGF binding proteins (IGFBPs). IGFs are secreted mostly by the liver and their secretion in adulthood is regulated by growth hormone (GH) and the nutritional status (Clemmons & Underwood 1991, Strauss 1994, Thissen et al. 1994). There is increasing evidence to suggest that thyroid hormones are intricately involved in the regulation of the GH/IGF axis at a number of levels (Rodriguez-Arnao et al. 1993). One of the most intriguing physiological events involving the IGF system in rats is that the fetal serum profile, characterized by high IGF-II and the 30 kDa complex of IGFBPs (IGFBP-1 and -2), is replaced around the third week of life by the adult-type profile of high IGF-1 and IGFBP-3, together with a dramatic reduction of IGF-II and the 30 kDa IGFBPs levels (Donovan et al. 1989). This shift in the serum profile is retarded by the lack of thyroid hormones (Gallo et al. 1991, Näntö-Salonen et al. 1991). Hypothyroid patients show low plasma IGF-I levels and reduced IGF bioactivity, whereas hyperthyroid patients present high plasma IGF-I levels and low IGF bioactivity (Miell et al. 1993); similar changes have been observed in rats (Burstein et al. 1979). Although plasma concentration and pituitary content of GH are regulated by thyroid hormones (Evans et al. 1982, DeFesi et al. 1984, Samuel et al. 1989), not all the effects of thyroid hormones on the IGF/IGFBP system are mediated by GH (Burstein et al. 1979, Ikeda et al. 1989), and the interrelationships between thyroid function and the IGF/IGFBP system are complex and not fully understood. Moreover, it has been suggested that the influence of thyroid hormones on this system is age dependent (Näntö-Salonen & Rosenfeld 1992).

In a model of thyroidectomized rats, it has recently been established that insulin mediates thyroid hormone effects on IGF secretion during the neonatal period while GH mediates those effects during the adult stage (Ramos et al. 1998). Moreover, a differential regulation of IGFBP-1 and -2 by insulin in thyroidectomized animals has been
suggested (Jones & Clemmons 1995, Rajaran et al. 1997). Since all the above studies were carried out in hypothyroid animals, only the study of rehabilitation of neonatal and adult thyroidectomized rats with thyroid hormone could provide new evidence to further understand the regulation of the IGF system by the thyroid hormones. At present, all the studies dealing with thyroid rehabilitation have been carried out by the injection of a single dose of thyroxine (T₄) during the neonatal or the adult period separately (Coiro et al. 1979, Nüttö-Salonen et al. 1991), but continuous replacement of thyroid hormone seems more appropriate. In the present study, we investigated the effect of different ways of replacing T₄ in thyroidectomized neonatal and adult rats on the circulating levels and liver mRNA expression of IGFs and their binding proteins IGFBP-1, -2, and -3, as well as on body weight, blood glucose, plasma insulin, thyrotropin (TSH) and GH and pituitary GH content. We started by studying the best method of thyroid hormone replacement in thyroidectomized rats at different age periods. Thyroxine replacement was carried out by two different methods of administration: 1) intermittent doses by daily injection, and 2) continuous replacement by pellet implantation.

In order to study the dose–response effect of thyroid hormones in neonatal rats, two different doses (1·5 and 3·5 µg) of T₄ were used; in adult rats, the same dose was administered for two different time periods, 5 and 10 days. The goals of the study were, first, to establish an appropriate method of administration of T₄ and to check whether the recovery of the alterations of the IGF/IGFBP system induced by thyroidectomy were T₄ dose dependent; secondly, to study the influence of the age of the animal on the specific action of T₄ on the IGF/IGFBP axis suggested previously and thirdly to clarify in vivo by means of a global study of thyroidectomized rats from the neonatal to the adult period, the potential direct effect of thyroid hormones on the regulation of IGF secretion which has previously been reported in vitro (Ikeda et al. 1989). A rigorous study of the recovery of circulating levels of thyroid hormones with different T₄ doses in thyroidectomized (Tx) rats from the neonatal and the adult stages, seemed necessary in order to contribute new data on the regulation of the IGF/IGFBP system by thyroid hormones in vivo.

Materials and Methods

Animals

Wistar rats bred in our laboratory under controlled temperature and an artificial light–darkness cycle (lights on 0600–1800 h) were used throughout the study. After birth, the number of pups in each litter was standardized to eight; males and females were used in equal numbers in neonatal populations, while only males were used for the adult groups. A standard laboratory diet was available ad libitum. Thyroidectomy and pellet implantation were performed under ether anesthesia, and control rats were sham operated. In order to prevent possible hypocalcemia from loss of the parathyroid glands after thyroidectomy, 1% calcium lactate was added to the drinking water of experimental and control rats. In order to investigate whether food intake was reduced in neonatal thyroidectomized animals, stomach milk content of Tx and control neonates was measured at 20 days of age. Blood was harvested from the trunk after decapitation and plasma or serum was stored at −80°C until assayed. Livers and pituitaries were frozen in liquid N₂.

All experiments were conducted in accordance with the principles and procedures outlined in the National Institutes of Health (NIH; Bethesda, Maryland, USA) guide for care and use of experimental animals.

Experimental models

Two populations of rats were used: neonatal (N) and adult (A). Rats were born (B), treated with mercapto-1-methylimidazole (MMI), thyroidectomized (Tx), replaced with T₄ (+T₄) and killed (K) at the indicated times. T₄ replacement was carried out by a daily i.p. injection (R) of 1·5 µg/100 g to neonates and of 1·75 µg/100 g to adults, or by pellet implantation with 1·5 µg/100 g (RP₁.₅) or 3 µg/100 g (RP₂) for 5 days in neonates, and 1·75 µg/100 g for 5 (RP₁.₅) or 10 (RP₂) days in adult rats. Control rats (C) were sham operated and left untreated.
implanted s.c. on day 15 with a T₄ pellet releasing 1·5 µg/100 g b.w. per day, and were killed on day 20. Tx₅+RP₅ animals were MMI-treated and Tx as above, were implanted s.c. on day 15 with a T₄ pellet releasing 3 µg/100 g b.w. per day, and were killed on day 20. Control rats (C) were sham operated and killed on day 20 (Fig. 1).

Adult rats were divided into five groups: Tx₇₂ animals received MMI (0·02% v/w added to the drinking water) from day 65 of life, were Tx on day 72 and were killed on day 87. MMI was given to ensure that T₃ and T₄ were adequately reduced. Tx₇₂+R animals were MMI-treated and Tx as above, were implanted s.c. on day 82 with a T₄ pellet releasing 1·75 µg/100 g b.w. T₄ dissolved in a slightly alkaline isotonic solution and were killed on day 87 of life. Tx₇₂+RP₅ animals were MMI-treated and Tx as above, and were implanted s.c. on day 82 with a T₄ pellet releasing 1·75 µg/100 g b.w. per day, and were killed on day 87. Tx₇₂+RP₁₀ animals were MMI-treated and Tx as above, implanted s.c. on day 82 with a T₄ pellet releasing 1·75 µg/100 g b.w. per day, and were killed on day 92; Control rats (C) were sham operated and killed on day 87 or day 92 (Fig. 1).

Due to the small size of the thyroid gland and low survival rate after thyroidectomy during the neonatal period, neonatal rats were thyroidectomized on day 5 of life, when the rat was still rather immature, and were killed on day 20 in order to carry out the neonatal study within the suckling period (22–23 days of life). Different T₄ doses were used in both neonatal and adult rats in order to find the minimal dose of thyroid hormones necessary to ensure a euthyroid condition in the thyroidectomized rats. Two different doses of T₄ were used during the neonatal period, 1·5 and 3 µg/100 g b.w. The latter dose was chosen because neonatal rats are reported to have greater thyroid hormone levels than adult rats, and a higher dose was necessary to ensure a euthyroid situation. The smaller dose was used to test the greater sensitivity of the neonatal animals to thyroid hormones. In order to control the euthyroid condition, plasma TSH was measured before and after T₄ treatment in Tx and control animals at both neonatal and adult stages. L-Thyroxine (T₄) and MMI were obtained from Sigma Chemical Co (St Louis, MO, USA). L-Thyroxine pellets were obtained from Innovative Research of America (Sarasota, FL, USA).

Serum glucose and plasma insulin, GH, TSH, T₃ and T₄ determinations

Glucose was determined with a Refloux IIM (Boehringer Mannheim, Leverkusen, Germany) glucose analyzer (Escrivá et al. 1992). Plasma immunoreactive insulin was estimated with purified rat insulin as standard (Novo, Nordisk Pharma, Madrid, Spain), antibody to porcine insulin, which cross-reacted similarly with pork and rat insulin standards, and moniodinated ¹²⁵I-labeled human insulin. The minimal detectable dose was 0·04 ng/ml, with a coefficient of variation within and between assays of 10%. Plasma and pituitary GH and plasma TSH were determined using the reagents which were generously distributed by the National Hormone and Pituitary Program of the NIDDK, NIH (rGH standard RP-2). The minimal detectable dose in pituitary homogenates and serum was 0·03 ng/ml GH. In order to prevent circadian variations in blood, samples were obtained during the same time window (between 1000 and 1200 h) any day from 6–8 animals. In order to avoid excessive manipulation of the animals, further sampling in Tx animals was discontinued. The coefficient of variation for the TSH assay was around 10%. Results are expressed as weight equivalents of the NIDDK rat TSH RP-3 reference preparation. Plasma T₃ and T₄ were determined at Centro de Investigaciones Biomédicas (CSIC) by highly specific RIAs as previously described (Weeke & Orskov 1975) and modified for rat plasma by Obregón et al. (1979). The minimal detectable doses were 2·5 pg for T₃ and 0·7 pg for T₄/assay tube.

Due to the large number of parameters measured in blood, samples from several rat neonates were pooled; this precluded the assay of each separate sample and hence the application of multivariance analysis. Therefore, all data were treated by ANOVA except the correlations which were analyzed by the multivariance analysis of the SPSS program.

Iodination, purification and determination of serum IGF-I and IGF-II

Recombinant human IGF-I and IGF-II were labeled by a modified Chloramine T method (Rivero et al. 1995). The specific activity achieved was approximately 90–175 µCi/µg for both peptides. Prior to IGF-I and -II determination, serum IGFBPs were removed by standard acid gel filtration. This method has proved to be the most reliable one for use with rat serum (Rivero et al. 1995).

The radioimmunoassay for IGF-I and the rat liver membrane receptor assay for IGF-II were carried out as previously described (Rivero et al. 1995). The coefficients of variation within and between assays were 8·0% and 12·4% respectively. Recombinant human IGF-I and -II (Boehringer Mannheim) were used for iodination.

Western ligand blotting

Western ligand blots were performed as previously described (Rivero et al. 1995, Goya et al. 1996). Briefly, sera were diluted in sample buffer (Tris–HCl, 0·625 M, pH 6·8; 10% (v/v) glycerol; 2% SDS and 0·0125% bromophenol blue); 2·5 µl serum were submitted to SDS-PAGE under non-reducing conditions (to prevent denaturation of IGFBPs) on the same 10% gel. After electrotransference to nitrocellulose, the membranes were incubated with ¹²⁵I-labeled IGF-II (10⁶ c.p.m.) for 20 h at 4 °C and autoradiographed against Hyperfilm MP.
between intensifier screens at −70 °C. Autoradiographs were quantified by two-dimensional densitometry using a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA, USA). Na125I and Hyperfilm–MP autoradiography film were obtained from Amersham (Amersham Ibérica SA, Madrid, Spain).

Western immunoblotting

Two different Western immunoblots were used: the enhanced chemiluminescence (ECL) method for neonatal rats and the alkaline phosphatase color reaction for adult rats. The latter was used because no IGFBP signal was obtained when goat polyclonal anti-rat IGFBP-1 and -2 ready for ECL were used in adult serum.

Western immunoblots for enhanced chemiluminescence were performed in polyvinylidene fluoride (PVDF) immobilon-P membranes (Millipore, Madrid, Spain). PVDF membranes were blocked with 5% (w/v) nonfat dry milk for 60 min in Tris–buffered saline (TBS, 0·01 M Tris, 0·15 M NaCl, pH 8) with 0·05% Tween-20. Membranes were then incubated with a 1:100 dilution (as suggested by the manufacturer) of affinity purified goat polyclonal anti-rat IGFBP-1 or rat IGFBP-2 (Santa Cruz Biotechnology, Quimigranel, Madrid, Spain) in the same buffer (TBS-Tween plus 5% nonfat dry milk) at 4 °C overnight, after which the membrane was washed three times for 10 min in TBS–Tween. After a 1–h incubation at room temperature with a 1:1000 dilution of anti-goat immunoglobulin G–horseradish peroxidase (IgG-HRP) in TBS–Tween plus 5% nonfat dry milk, the membrane was washed three times with TBS–Tween and finally once with TBS alone. Antigen–antibody complexes were detected following an enhanced chemiluminescence (hyperfilm ECL, Amersham Ibérica SA).

For the alkaline phosphatase color reaction, Western immunoblots were performed in the same membranes as Western ligand blots. This method has previously been described in detail (Rivero et al. 1995, Goya et al. 1996). Since densitometric quantification of the color reaction in nitrocellulose membranes was not available, this method was valid only for comparing band intensities, thus quantitative results are not shown.

Preparation of RNA

Total RNA was prepared by homogenization of livers in guanidinium thiocyanate as originally described (Goya et al. 1996). Samples were electrophoresed through 1% agarose, 2·2 mol formaldehyde/l gels and stained with ethidium bromide in order to visualize the 28S and 18S ribosomal RNA and thereby confirm the integrity of the RNA. pT7 RNA 18S antisense control template (Ambion Inc, Austin, TX, USA) was used to normalize the quantity of RNA in the different lanes.

Riboprobes

Rat IGF-I and -II and IGFBP-1, -2 and -3 cDNAs were kindly provided by Drs C T Roberts Jr and D LeRoith (NIH) (see Goya et al. 1996 for details of the preparation of riboprobes for IGF-I, -II, IGFBP-1, -2 and −3). [32P]UTP was purchased from ICN (Nuclear Ibérica S.A., Madrid, Spain). Riboprobe Gemini II Core System (Promega Corporation, Madison, WI, USA) was used for the generation of RNA probes.

Solution hybridization/RNase protection assay

Solution hybridization/RNase protection assays were performed as previously described (Goya et al. 1996, Ramos et al. 1998). Autoradiography was performed at −70 °C against a Hyperfilm–MP film between intensifying screens. Bands representing protected probe fragments were quantified using a Molecular Dynamics scanning densitometer and accompanying software. RNase A and RNase T1 were purchased from Boehringer Mannheim.

Statistical analysis

All data in the figures are presented as means ± s.e. Statistical comparisons were performed by one-way analysis of variance (ANOVA), followed by the protected least significant difference test. Correlations between different variables and partial correlations were calculated with the SPSS program for Macintosh version 6·1·1. (SPSS Chicago II). P<0·05 was considered as significant.

Results

Stomach content in neonates and body weight, circulating T3, T4, TSH, IGFs, insulin and GH levels, pituitary GH content and liver RNA expression of IGFs in thyroidectomized neonatal and adult rats treated with T4: correlations and partial correlations (Tables 1 and 2, and Figs 2 and 3)

Food intake, as measured by the milk content in stomach, was similar in 20-day-old Tx and control neonates (0·64 ± 0·19 and 0·85 ± 0·22 g respectively). No significant differences were observed when the stomach content/body weight ratio was calculated for both groups and compared.

Table 1 shows the decreased body weight, serum T3 and T4 and blood glucose and the increased plasma TSH, GH and insulin and pituitary GH content in Tx neonatal rats compared with controls. After a daily injection of T4 (1·5 μg/100 g b.w.) for 5 days (Tx5+R), body weight of Tx rats increased but remained below that of controls. Body weight increased to reach control values in Tx5+R and increased further in Tx5+RP1,5, whereas plasma TSH remained higher than controls. A complete
Table 1 Body weight, T₃, T₄, GH, TSH, insulin plasma levels, GH pituitary content and glycemia in thyroidectomized (Tx) neonatal rats treated with T₄ by i.p. injection (Tx₅+R) or by s.c. pellet (Tx₅+RP₁₋₅) with the same dose of 1.5 µg/100 g b.w. each day or by pellet with a dose of 3 µg/100 g b.w. each day (Tx₅+RP₃) for 5 days. Rats were killed at 20 days of age. Results are means ± s.d., n=8-10, except Tx₅+RP₅, n=5.

<table>
<thead>
<tr>
<th>Body weight (g)</th>
<th>Serum T₃ (ng/dl)</th>
<th>Serum T₄ (µg/dl)</th>
<th>Plasma TSH (ng/ml)</th>
<th>Plasma GH (µg/ml)</th>
<th>Pituitary GH (µg/mg)</th>
<th>Blood glucose (mg/100 ml)</th>
<th>Plasma insulin (µU/ml)</th>
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<tbody>
<tr>
<td>Tx₅ Controls</td>
<td>23.16 ± 0.83</td>
<td>6.98 ± 0.02</td>
<td>12.3 ± 0.066</td>
<td>131.5 ± 67.3</td>
<td>7.59 ± 0.62</td>
<td>15.49 ± 3.54</td>
<td>104.25 ± 7.56</td>
</tr>
<tr>
<td>Tx₅+R</td>
<td>32.61 ± 1.82</td>
<td>90.52 ± 1.06</td>
<td>14.1 ± 0.45</td>
<td>161.2 ± 65.8</td>
<td>7.1 ± 0.06</td>
<td>138.5 ± 145</td>
<td>132.50 ± 2.98</td>
</tr>
<tr>
<td>Tx₅+RP₁</td>
<td>25.75 ± 2.57</td>
<td>30.49 ± 0.88</td>
<td>24.8 ± 0.049</td>
<td>161.2 ± 65.8</td>
<td>32.1 ± 3.9</td>
<td>210.5 ± 0.47</td>
<td>133.91 ± 1.62</td>
</tr>
<tr>
<td>Tx₅+RP₃</td>
<td>39.85 ± 1.49</td>
<td>90.10 ± 0.82</td>
<td>7.40 ± 0.047</td>
<td>161.2 ± 65.8</td>
<td></td>
<td>26.87 ± 3.58</td>
<td>134.25 ± 2.26</td>
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</table>

*P<0.05 relative to C rats; *P<0.05 relative to Tx₅ rats; **P<0.05 relative to Tx₅+R rats; ***P<0.05 relative to Tx₅+RP₅ rats.

These results show that the best recovery of circulating thyroid hormones and IGF-I to steady-state levels after T₄ treatment was observed in the Tx₀+RP₁₀ group and that overall pellet implantation was a better treatment than injection. Decreased body weight and liver mRNA levels and protein content of IGF-I after T₄ and IGF-I expression after T₄, but not after T₃, were better in those of control animals. In agreement, a significant reduction of the plasma TSH levels only decreased to control values in Tx₀+RP₅, the group in which plasma TSH levels only decreased to control values in Tx₀+RP₅. Plasma GH did not reach control values in Tx₀+RP₅ and Tx₀+RP₆, although serum T₃, T₄, and TSH levels approached or were similar to those of controls, while plasma insulin was not altered in Tx₀ and Tx₀+R and Tx₀+RP₅.

Although increases in body weight, plasma GH and insulin and primary GH content were observed after the three T₄ treatments, the values reached in Tx₀+RP₂ and Tx₀+RP₅ were lower than those of Tx₀+RP₅. In agreement, a significant reduction of the plasma TSH levels only decreased to control values in Tx₀+RP₅, the group in which plasma TSH levels only decreased to control values in Tx₀+RP₅.

In agreement, a significant reduction of the plasma TSH levels only decreased to control values in Tx₀+RP₅ and Tx₀+RP₆. In agreement, a significant reduction of the plasma TSH levels only decreased to control values in Tx₀+RP₅ and Tx₀+RP₆. In agreement, a significant reduction of the plasma TSH levels only decreased to control values in Tx₀+RP₅ and Tx₀+RP₆. In agreement, a significant reduction of the plasma TSH levels only decreased to control values in Tx₀+RP₅ and Tx₀+RP₆. In agreement, a significant reduction of the plasma TSH levels only decreased to control values in Tx₀+RP₅ and Tx₀+RP₆.
Unfortunately, we have not been able to apply a more appropriate multivariance analysis with the SPSS program to our data, as it has not been possible to measure all variables in blood samples individually, especially in neonates. Therefore, the data were analyzed by ANOVA, except correlations and partial correlations that were calculated with the SPSS multivariance program for Macintosh. It was found that in neonatal rats, body weight was not correlated to IGF-I or IGF-II. However, body weight was correlated to insulin and glucose levels ($P=0.031$ and $P=0.038$ respectively); IGF-II, the most abundant IGF in the perinatal stages, correlated to insulin even when corrected for $T_3$ and $T_4$ ($P=0.001$), and with

![Figure 2](image-url)

**Figure 2** (A) Serum IGF-I by RIA and IGF-II by radioreceptor assay in neonatal thyroidectomized (Tx), control (C) and Tx treated with $T_4$ (1-5 mg/100 g b.w. (RP$_{1.5}$) or 3 mg/100 g b.w. per day (RP$_{3}$)) by pellet implantation. Plasma from 3–4 different rats was pooled and eight different pools per group were used for the assay. (B) Liver mRNA expression of IGF-I and IGF-II by RNase protection assay (RPAs) in the same populations. Representative bands for each transcript are shown in the figure. Densitometric quantification of all bands from the same group is also shown as arbitrary units. Four different samples per group were assayed in two separate RPAs. Eight samples from different animals were used in the assay. All rats were killed 15 days after Tx at 20 days of age (15aTx (20 D)). Results are means $\pm$ S.D., *$P<$0.05 relative to control rats; $P<$0.05 relative to Tx rats; $P<$0.05 relative to $T_4$ rats.

### Table 2

<table>
<thead>
<tr>
<th>Body weight (g)</th>
<th>Serum $T_3$ (ng/dl)</th>
<th>Serum $T_4$ (ng/dl)</th>
<th>Plasma TSH (ng/ml)</th>
<th>Plasma GH (ng/ml)</th>
<th>Pituitary GH (ng/mg)</th>
<th>Blood glucose (mg/100 ml)</th>
<th>Plasma insulin (U/ml)</th>
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<tr>
<td>Controls</td>
<td>16.70±2.35</td>
<td>9.45±0.30</td>
<td>4.94±0.45</td>
<td>4.16±1.02</td>
<td>9.42±3.48</td>
<td>13.1±2.11</td>
<td>14.6±2.98</td>
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<tr>
<td>Tx2</td>
<td>19.40±0.50</td>
<td>17.49±0.45</td>
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<tr>
<td>Tx2+RP$_{1.5}$</td>
<td>18.72±0.50</td>
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<tr>
<td>Tx2+RP$_3$</td>
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thyroid hormones, even when corrected for insulin ($P = 0.001$ for T4 and $P = 0.041$ for T3). Blood glucose levels were not only correlated to body weight ($P = 0.038$), but also to thyroid hormones ($P = 0.045$ for T3 and $P = 0.038$ for T4). On the contrary, in adults, no correlations were found among insulin or blood glucose or any of the other variables. IGF-I, the most abundant IGF in adults, correlated with T3 ($P = 0.001$) and with body weight ($P = 0.046$). T3 and T4 levels were highly correlated ($P < 0.001$) in both the neonatal and adult rats ($P < 0.05$ was considered significant).

**Figure 4** (A) Western ligand blot of neonatal rats thyroidectomized (Tx), control (C) and Tx treated with T4 by pellet implantation (RP10) (1.5 μg/100 g b.w.) for 5 days. (B) Western immunoblot of IGFBP-1 and IGFBP-2 in the same populations, to evaluate the IGFBP-1 and -2 ratio in the 30 kDa complex tested by Western ligand blot. All rats were killed 15 days after Tx (aTx), at 20 days of age (20 D). Representative bands are shown in the figure. Densitometric quantification of all bands from the same group is also shown as arbitrary units. Sera from 3–4 different animals were pooled and six different pools per group were used for the assay. Results are means ± s.d.

* $P < 0.05$ relative to control rats; ▲ $P < 0.05$ relative to Tx rats.

IGF recovery in thyroidectomized rats

**Figure 3** (A) Serum IGF-I in adult rats thyroidectomized (Tx), control (C) and Tx treated with T4 (1.75 μg/100 g b.w. each day) by i.p. injection (R) or s.c. pellet for 5 (RP5) or 10 days (RP10). All groups were killed 15 days after Tx on day 87 except for RP10 group which was killed at 92 days of life (15–20 aTx (87–92 D)) (see Fig. 1). Sera of six different animals were analyzed. (B) Liver mRNA expression of IGF-I in the same adult populations. Transcript levels were determined by RNase protection assay (RPA). A representative experiment of bands of RNase protection assay with the specific probe is depicted in the figure. Densitometric quantification of all bands from the same group is also shown as arbitrary units. Two different samples per group were assayed in three separate RPAs with a total of six animals analyzed. Results are means ± s.d. * $P < 0.05$ relative to control rats; ▲ $P < 0.05$ relative to Tx rats.

Western ligand blot and immunoblot of serum IGFBP levels and liver mRNA expression of IGFBPs in thyroidectomized neonatal and adult rats treated with T4 (Figs 4, 5 and 6)

Western ligand blot showed that the 45 kDa band of IGFBP3 (IGFBP-3) decreased and the 30 kDa complex of IGFBPs (IGFBP-1 and -2) increased in Tx neonatal rats compared with controls (Fig. 4A). Specific Western immunoblot depicted in Fig. 4B shows that the increase in the 30 kDa complex resulted from the rise of both IGFBP-1 and IGFBP-2. A recovery of the 30 kDa complex, especially IGFBP-2 (Fig. 4B), and the 45 kDa band (Fig. 4A) was already observed in Tx$_5$+RP$_{10}$; therefore, a greater dose of T$_4$ was not assayed. Parallel to thyroid hormones, even when corrected for insulin ($P = 0.001$ for T$_4$ and $P = 0.041$ for T$_3$). Blood glucose levels were not only correlated to body weight ($P = 0.038$), but also to thyroid hormones ($P = 0.045$ for T$_3$ and $P = 0.038$ for T$_4$). On the contrary, in adults, no correlations were found among insulin or blood glucose or any of the other variables. IGF-I, the most abundant IGF in adults, correlated with T3 ($P = 0.001$) and with body weight ($P = 0.046$). T$_3$ and T$_4$ levels were highly correlated ($P = 0.001$) in both the neonatal and adult rats ($P < 0.05$ was considered significant).
the changes found in serum, Fig. 5 shows that Tx neonatal rats presented an increase in the liver mRNA expression of IGFBP-1 and -2 and a decrease in the gene expression of IGFBP-3, suggesting a transcriptional regulation of these proteins in the liver. Liver mRNA expression of IGFBP-1, -2 and -3 recovered in Tx5+RP1·5 and Tx5+RP3, but IGFBP-2 transcripts reached levels below those of controls in Tx5+RP1·5 and were even lower in Tx5+RP3. The results obtained after T4 administration also paralleled in part those of serum levels (Fig. 4), supporting the transcriptional regulation of the three proteins in the neonatal period. The results show that recovery of Tx neonates with T4 evoked dose-dependent changes in liver mRNA expression of IGFBPs. In particular, during the neonatal period, the rise in liver mRNA expression of IGFBP-2 evoked by thyroid hormone deficiency was reduced by T4 administration (Figs 4 and 5).

A decrease in the 45 kDa band and the 30 kDa complex of IGFBPs in Tx adult rats compared with controls can be observed in Fig. 6A. Specific Western immunoblot depicted in Fig. 6B shows the increase in IGFBP-2 in Tx adult rats and the decrease of this protein after all T4 treatments. Unfortunately, reliable densitometric values from this immunoblot assay could not be obtained with the means available. In adult rats (Fig. 6A), a complete recovery of both IGFBP-3 (45 kDa) and the 30 kDa complex was achieved only in Tx72+RP10.

In adult rats, all T4 treatments recovered liver mRNA expression of IGFBP-1, -2 and -3, but an increase of IGFBP-1 transcripts above the control values was observed in Tx72+R (Fig. 5). IGFBP-2 decreased below the levels of controls in Tx72+R and Tx72+RP5, and IGFBP-3 increased to values above those of controls in Tx72+RP5 and Tx72+RP10. Thus, the results depicted in Figs 5 and 6 suggest that all three IGFBPs are transcriptionally regulated in the adult liver under these conditions. Although liver mRNA expression of the main IGFBPs (IGFBP-3) was generally recovered in Tx72+R, a complete recovery of the serum levels of IGFBPs was obtained only in Tx72+RP10, in which the euthyroid condition was attained. These results confirm that pellet implantation for 10 days (Tx72+RP10) is the most suitable T4 treatment for recovery of the IGF/IGFBP system in Tx adult rats. Furthermore, similar to what was found in neonates, thyroid hormone deficiency induced a rise in serum levels and liver mRNA expression of IGFBP-2 which was reduced by T4 administration (Figs 5 and 6).

Discussion

Influence of T4 dose and method of administration

Administration of T4 by bolus injection has been the preferred method for thyroid rehabilitation in studies on the regulation of the IGF/IGFBP system in hypothyroid animals (Coiro et al. 1979, Näntö-Salonen et al. 1991).
However, studies on TSH regulation in thyroidectomized rats have recommended continuous administration of thyroid hormone by pellet implantation (Connors & Hedge 1981). The present results show that the replacement of thyroid hormone necessary to ensure a recovery of the IGF/IGFBP system is achieved only by T4 pellet implantation, when the euthyroid condition was established. In this study, T4 treatment by injection (R) induced transient changes, such as an increase in liver mRNA expression of IGFBP-1 in adults, which were not maintained. The effect of increasing doses and length of treatment of T4 is shown in the progressive recovery of serum and liver mRNA expression of IGF-I observed in neonatal rats treated with 1·5 and 3 µg T4 compared with controls (Fig. 2), as well as in adult rats from Tx72+R to Tx72+RP10 (Fig. 3). Likewise, plasma TSH data show that the euthyroid condition is only attained after treatment of neonates with a 3 µg pellet and a 10-day treatment of adults (Tx72+RP10). Furthermore, the dose-dependent recovery can also be observed in the rest of the parameters included in Tables 1 and 2 from neonatal and adult Tx rats treated with T4, a finding that had not been reported previously because a single dose of thyroid hormone was used to restore the euthyroid condition. All the above suggests a dose–response effect of serum thyroid hormones on IGF secretion in live animals, an effect previously reported in vitro (Ikeda et al. 1989). The different responses observed between Tx and control neonates could not have been caused by a different nutritional status, since the stomach milk content was similar in both groups. The direct influence of T4 has also been suggested by the good correlation found between thyroid hormone and IGF-II in neonates and thyroid hormone and IGF-I in adults. During the neonatal period, the relevant role of thyroid hormones is shown by the reduced body weight in Tx rats despite the elevated circulating GH, IGF-I and insulin. Likewise, the developmental pattern of the endocrine system during the neonatal period could be an explanation for the unexpected increase in plasma and pituitary GH and plasma insulin found in Tx neonatal rats compared with controls but this seems unlikely since similar results have not been observed in MMI-induced hypothyroid neonatal rats (Ramos et al. 1998). However, a decreased milk content in the stomach of MMI-hypothyroid animals, compared to that observed in Tx neonatal rats, has recently been observed (unpublished data from our laboratory) probably due to the sour taste of milk from mothers of MMI-hypothyroid rats receiving 0·05 w/v MMI (Ramos et al. 1998) as opposed to 0·02 w/v given to mothers of Tx neonates. The differential nutritional status between the two groups could explain the distinct response of insulin, GH and IGFs in both populations as compared with controls. Notwithstanding, the above changes could result from thyroid withdrawal-induced variations in catecholamine and/or glucocorticoid levels (Kitabchi et al. 1968, Smith & Porte 1976, Aránguez et al. 1986), as well as from

Figure 6 (A) Western ligand blot (WLB) of adult rats thyroidectomized (Tx), control (C) and Tx treated with T4 (1·75 µg/100 g b.w.) by i.p. injection (R) or by pellet implantation for 5 (RP5) or 10 days (RP10) (see Fig. 1). (B) Western immunoblot specific for IGFBP-2 in the same populations to quantitate IGFBP-2 within the 30 kDa complex. Representative bands are shown in the figure. Densitometric quantification of all bands from the same group is also shown as arbitrary units. Two different samples per group were assayed in two separate WLBs with a total of 4–6 animals analyzed. Results are means ± s.d. *P<0·05 relative to control rats; ▲P<0·05 relative to Tx rats; ●P<0·05 relative to R rats.
the subtle balance between thyroid hormones and insulin since both increases and decreases (Lenzen & Bailey 1984) of insulin have been reported in hypothyroid situations, probably depending on the degree of thyroid hormone deprivation.

**Regulation of the IGF/IGFBP system in neonatal thyroidectomized rats treated with T4**

The utilization of the smallest dose of T4 in Tx neonatal rats showed the high sensitivity of the neonatal pituitary, since the rapid increase in pituitary and serum GH found with this dose in neonates was found in adults only after administration of a higher dose (1.75 µg). However, the euthyroid condition and complete recovery of liver mRNA of IGF-I was achieved in Tx neonates only after administration of the higher dose of T4 (3 µg). External doses of T4 (1.5 or 3 µg) decrease IGFs in neonates in parallel to the dose–response decrease in insulin and despite the T4-induced increase in GH (Table 1). The latter results from a well known direct effect of thyroid hormones at the pituitary, and T4 administration increased GH levels well above those of controls, since serum GH levels were already elevated in Tx neonates. All the above suggest the relevant effect of the condition of the thyroid on the regulation of IGFs throughout thyroid hormone–induced changes in insulin secretion. Besides, the results suggest that regulation of IGFs in neonates is GH independent, according to the calculated correlations, and seems to be controlled by the thyroid status of the animal. Elevated serum levels and liver mRNA expression of IGF-II have previously been found in Tx neonatal rats (Ramos et al. 1998), and the present results show that low T4 doses (1.5 µg) recovered blood glucose, decreased serum insulin and reduced both serum levels and liver mRNA expression of IGF-II, in agreement with the good partial correlation found between IGF-II and insulin in this neonatal population, a finding that has not been reported previously. Correlation and partial correlation, by multivariate analysis, support the conclusion that body weight and serum IGF-II are regulated by insulin, glucose and thyroid hormones during the neonatal period, in concert with the effect of insulin on the regulation of IGFs reported in undernourished and diabetic neonatal populations (Rivero et al. 1995, Goya et al. 1996). All the above, together with results obtained in hypophysectomized infant rats (Glasscock & Nicoll 1981), suggest a crucial role of thyroid hormones in the regulation of growth during the immature stages.

IGFBPs regulate IGF bioavailability, and their secretion is controlled by different hormones and by serum IGF-I levels depending on the physiological situation (Rajaran et al. 1997). The results obtained after recovery of serum levels and liver mRNA expression of IGFBPs in these neonatal rats show a direct correlation with the dose and way of administration of T4. The results also show that, although the small dose of T4 administered by pellet (RP1) to Tx neonates did not lead to the recovery of liver mRNA expression of IGF-I, nor the euthyroid status, it did increase serum and liver mRNA expression of the 30 kDa complex of IGFBPs (the most abundant in the neonatal period) and also of IGFBP-3 and restored them to control values. This novel result suggests that IGFBPs are more sensitive to exogenous T4 doses than IGF-I during the neonatal period.

Elevated insulin observed in Tx neonatal rats decreased after T4 replacement but did not reach control levels, while no changes in serum IGFBP-1 were observed in Tx+RP1 despite the well known insulin-induced down-regulation of this protein (Holly et al. 1988, Lewitt et al. 1994). However, it has been reported that changes in IGFBP-1 in children may not be limited specifically to changes in insulin secretion (Orlowski et al. 1990, Counts et al. 1992, Bereket et al. 1995, Smith et al. 1995, Strasser-Vogel et al. 1995), a finding that supports the results obtained in the present study in T4-treated Tx neonatal rats, and suggests that the regulation of IGFBP-1 by insulin might take place only in conditions of severe insulinopenia (Ooi et al. 1990, Muñoz et al. 1996). RNase protection assay of liver showed that T4 treatment of Tx neonatal rats induced a dose–dependent decrease in IGFBP-2. Thus, the results obtained after T4 replacement of Tx neonatal rats indicated, as previously suggested (Näntö-Salonen et al. 1991), that a direct effect of circulating thyroid hormones on IGFBP-2 cannot be ruled out. The decrease in IGFBP-3 observed in Tx neonatal rats takes place in the presence of elevated GH, insulin and IGF-I, which could suggest a direct effect of thyroid hormone deprivation in the neonatal period. But in the present study, low doses of T4 administered to Tx neonates (1.5 µg) evoked a further increase in plasma GH and increased serum and liver mRNA expression of IGFBP-3 to control values, supporting the idea that the T4-induced rise in GH mediates IGFBP-3 synthesis and secretion in 22-day-old Tx neonatal rats, as occurs in adult rats. The latter results show the role of thyroid function in the regulation of IGFBP-3.

**Regulation of the IGF/IGFBP system in thyroidectomized adult rats treated with T4**

As previously described (Ramos et al. 1998), a decrease in circulating thyroid hormones, GH and insulin was found in Tx adult rats. After T4 administration, the progressive recovery of the metabolic and endocrine parameters observed in adult rats from Tx2+R to Tx2+R16, more evident than in neonates treated with increasing doses of T4, seems to indicate the maturity of the regulatory pattern of the endocrine system (Glydon 1975, Walker et al. 1977). The parallel recovery of liver mRNA expression and serum levels of IGF-I in Tx adult rats treated with T4 suggests a transcriptional regulation of the gene. Contrary to what is found in neonatal rats, the relationships between
measured variables in adults, based on correlation and partial correlation by a multivariate analysis, show that serum levels of thyroid hormones, rather than insulin and glucose, influence body weight and IGF-I. This fact also indicates the lack of correlation between serum insulin and IGF-I in adult rats, previously suggested also in undernourished and diabetic adult rats (Goya et al. 1996). In adult animals, lack of thyroid hormones results in a decrease in insulin and GH and, consequently, in IGF-I, showing its GH-dependency in mature stages. The reduction of insulin after Tx in adults, contrary to the increase found in the same conditions in neonates, shows the differential adaptation of insulin secretion to Tx depending on age. A different adaptation between diabetic neonates and adults has also been found in the liver thyroid status in our laboratory (data not shown). All the above seem to suggest that a balance between insulin and thyroid hormones is necessary for the regulation of growth in the immature stages.

Thyroidectomy induced a decrease in the 30 kDa complex of IGFBPs and IGFBP-3 in adult rats. RP10 stands out as the most suitable method for T4 restoration, and a progressive recovery of all IGFBPs from R to RP10 adult rats was found. The parallel recovery of liver mRNA expression and serum concentrations of IGFBP-3, the most abundant IGFBP in adulthood, after T4 replacement of Tx adult rats suggests a transcriptional regulation of the protein, probably mediated by rises in plasma GH and serum IGF-I induced by T4 treatment, a regulatory pattern previously reported (Jones & Clemmons 1995, Ramos et al. 1998). The T4-induced increase in the serum 30 kDa complex of IGFBPs in Tx adult rats to control levels resulted from increased liver mRNA expression of IGFBP-1 and decreased expression of IGFBP-2. Therefore, IGFBP-1 down-regulation by insulin was not found in adult rats, as in neonates, since insulin decreased after Tx and increased after T4 replacement, changes which parallel those of IGFBP-1. This result shows that insulin down-regulates IGFBP-1 in vivo only under conditions of severe diabetes, as stated in the neonatal period (Ooi et al. 1990). Thyroidectomy increased liver mRNA expression of IGFBP-2 in adult rats in conditions of low insulin, and T4 replacement reduced liver mRNA expression of IGFBP-2 in parallel with an increase in serum insulin. These results suggest a reverse correlation between thyroid hormones and IGFBP-2 in adult rats, a finding previously reported only for the neonatal period (Nánto-Salonen et al. 1991). Moreover, Western immuno blot and RNase protection assay showed increased IGFBP-2 in Tx adult rats and its reduction after T4 replacement, suggesting a transcriptional regulation of the protein.

Final considerations
These results suggest that insulin seems to be, together with the thyroid hormones, very decisive in the regulation of IGF-II secretion during the neonatal period, whereas no correlation was found between insulin and IGF-I in adults. The data also suggest a greater effectiveness of thyroid hormone action in recovering IGFBPs compared with IGFs, in particular during the neonatal period, a finding previously unreported. In summary, three final points can be concluded from these results. First, when studying the regulation of the IGF/IGFBP system by thyroid hormones, continuous T4 replacement by pellet is the only suitable procedure to follow in the rehabilitation of thyroidectomized animals since a complete euthyroid status, as measured by the TSH levels, was only attained after such procedure and with the highest doses utilized. Secondly, the results of this global study including neonatal and adult Tx rats before and after T4 administration, show a differential mechanism of recovery of the IGFBPs/IGFBPs system in Tx rats treated with T4 depending on the age period considered, probably due to the different changes of insulin after thyroidectomy, since serum GH changes in parallel with insulin, as previously reported (González & Jolin 1985). Finally, these experiments in vivo demonstrate that IGFBPs decrease after T4 administration to Tx neonatal rats despite the rise in GH, showing that in immature stages insulin, rather than GH, regulates IGFBPs. Besides, the results obtained suggest a possible direct effect of thyroid hormones on the IGF regulation in vivo since a complete recovery of IGFBPs is observed only when the euthyroid condition has been ensured, although further research is needed to unravel the mechanism.

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