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 (T_4) delivered by injection or s.c. pellet implantation on alterations of the IGF/IGF binding protein (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 T_4 (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 T_4 (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 T_4 administration indicated a high sensitivity to T_4 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 T_4 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 T_4 but not between insulin and IGF-I in Tx adults. IGFBP-2 seems to be up-regulated by T_4 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 T_4 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-I 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

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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 (T_4) during the neonatal or the adult period separately (Coiro *et al.* 1979, Näntö-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 T_4 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 μg) of T_4 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 T_4 and to check whether the recovery of the alterations of the IGF/IGFBP system induced by thyroidectomy were T_4 dose dependent; secondly, to study the influence of the age of the animal on the specific action of T_4 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 T_4 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 N_2.

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.

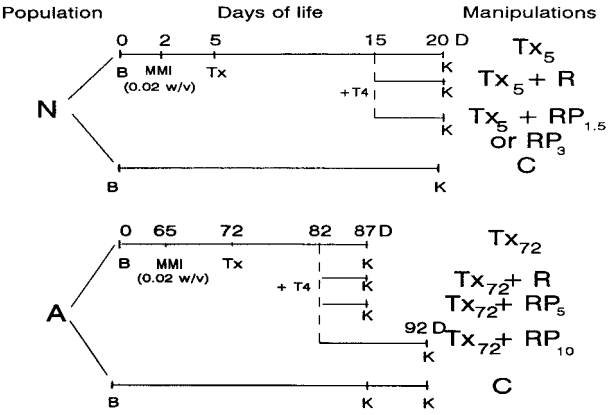

Figure 1 Two populations of rats were used: neonatal (N) and adult (A). Rats were born (B), treated with mercapto-1-methylimidazole (MMI), thyroidectomized (Tx), replaced with T_4 (+T4) and killed (K) at the indicated times. T_4 replacement was carried out by a daily i.p. injection (R) of 1·5 μg/100 g to neonates and of 1·75 μg/100 g to adults, or by pellet implantation with 1·5 μg/100 g ($RP_{1·5}$) or 3 μg/100 g (RP_3) for 5 days in neonates, and 1·75 μg/100 g for 5 (RP_5) or 10 (RP_{10}) days in adult rats. Control rats (C) were sham operated and left untreated.

Experimental models

Two populations of rats were used: neonatal and adult (Fig. 1). Neonatal rats were divided into five groups: Tx_5 animals received mercapto-1-methylimidazole (MMI) (0·02% w/v added to the drinking water of the mother) from day 2 of life and were Tx on day 5 of life. MMI was given to ensure that tri-iodothyronine (T_3) and T_4 were adequately reduced and to produce thyroid hyperplasia in order to facilitate thyroid gland withdrawal in 5-day-old neonates. Tx_5+R: animals MMI-treated and Tx as above received, from day 15 on, a daily injection of 1·5 μg/100 g body weight (b.w.) T_4 dissolved in a slightly alkaline isotonic solution and were killed on day 20 of life. Tx_5+$RP_{1·5}$: animals MMI-treated and Tx as above, were

implanted s.c. on day 15 with a T_4 pellet releasing 1·5 μg/100 g b.w. per day, and were killed on day 20. Tx_5+RP_3: animals MMI-treated and Tx as above, were implanted s.c. on day 15 with a T_4 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: Tx_{72} animals received MMI (0·02% w/v 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 T_3 and T_4 were adequately reduced. Tx_{72}+R animals were MMI-treated and Tx as above and received, from day 82 on, a daily injection of 1·75 μg/100 g b.w. T_4 dissolved in a slightly alkaline isotonic solution and were killed on day 87 of life. Tx_{72}+RP_5 animals were MMI-treated and Tx as above, and were implanted s.c. on day 82 with a T_4 pellet releasing 1·75 μg/100 g b.w. per day, and were killed on day 87. Tx_{72}+RP_{10} animals were MMI-treated and Tx as above, implanted s.c. on day 82 with a T_4 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 T_4 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 T_4 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 T_4 treatment in Tx and control animals at both neonatal and adult stages. L-Thyroxine (T_4) 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, T_3 and T_4 determinations

Glucose was determined with a Reflolux 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 monoiodinated ^{125}I-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 T_3 and T_4 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 T_4 and 0·7 pg for T_3/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 ^{125}I-labeled IGF-II (10^6 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). $Na^{125}I$ 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). [^{32}P]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.D. 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 T_3, T_4, TSH, IGFs, insulin and GH levels, pituitary GH content and liver RNA expression of IGFs in thyroidectomized neonatal and adult rats treated with T_4: 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 T_3 and T_4 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 T_4 (1·5 μg/100 g b.w.) for 5 days (Tx_5+R), body weight of Tx rats increased but remained below that of controls. Body weight increased to reach control values in Tx_5+RP_3. Serum T_3 and T_4 increased after T_4 administration in Tx_5+R and increased further in Tx_5+$RP_{1·5}$, whereas plasma TSH remained higher than controls. A complete

Table 1 Body weight, T_3, T_4, GH, TSH, insulin plasma levels, GH pituitary content and glycemia in thyroidectomized (Tx) neonatal rats treated with T_4 by i.p. injection (Tx_5+R) or by s.c. pellet ($Tx_5+RP_{1·5}$) 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 (Tx_5+RP_3) for 5 days. Rats were killed at 20 days of age. Results are means ± S.D., n=8–10, except Tx_5+RP_3, n=5

	Body weight (g)	Serum T_3 (ng/dl)	Serum T_4 (μg/dl)	Plasma TSH (ng/ml)	Plasma GH (ng/ml)	Pituitary GH (μg/mg)	Blood glucose (mg/100 ml)	Plasma insulin (μU/ml)
Tx_5	23·16 ± 0·83[a]	6·98 ± 0·02[a]	1·23 ± 0·06[a]	131·5 ± 67·3[a]	7·59 ± 0·62[a]	15·49 ± 3·54[a]	104·25 ± 7·56[a]	101·21 ± 12·08[a]
Controls	41·55 ± 0·71	90·52 ± 1·06	6·85 ± 0·34	7·2 ± 0·2[b,c,d]	2·18 ± 0·18	13·85 ± 1·45	132·50 ± 2·98	34·96 ± 3·12
Tx_5+R	32·61 ± 1·82[a,b]	15·15 ± 2·43[a]	1·41 ± 0·05[a]	195·0 ± 41·5[a]	14·07 ± 2·55[a,b]	20·15 ± 0·47[a,b]	114·93 ± 2·95[a]	62·31 ± 10·08[a,b]
$Tx_5+RP_{1·5}$	25·75 ± 2·57[a,c]	30·49 ± 0·88[a,b,c]	2·48 ± 0·09[a,b,c]	161·2 ± 65·8[a]	24·24 ± 3·53[a,b,c]	26·87 ± 3·58[a,b,c]	133·91 ± 1·62[b,c]	86·43 ± 7·29[a,b,c]
Tx_5+RP_3	39·85 ± 1·49[b,c,d]	90·10 ± 0·82[b,c,d]	7·40 ± 0·47[b,c,d]	7·1 ± 0·6[b,c,d]	32·1 ± 3·9[a,b,c]	35·8 ± 4·6[a,b,c]	134·25 ± 2·26[b,c]	77·35 ± 14·18[a,b,c]

[a]P<0·05 relative to C rats; [b]P<0·05 relative to Tx_5 rats; [c]P<0·05 relative to Tx_5+R rats; [d]P<0·05 relative to $Tx_5+RP_{1·5}$ rats.

recovery of serum T_3, T_4 and plasma TSH to control values was found only in Tx_5+RP_3 animals. A further increase in plasma GH and pituitary GH content to values above those of controls was observed in $Tx_5+RP_{1·5}$ and they increased still further in Tx_5+RP_3. Blood glucose increased after all T_4 treatments of Tx neonatal rats and reached control levels in $Tx_5+RP_{1·5}$ and Tx_5+RP_3. Finally, insulin decreased after all T_4 treatments but the values remained well above those of control rats, even in Tx_5+RP_3, in which the values were closer to controls. Plasma TSH decreased to control values, and therefore euthyroid status was attained, only after treatment with the 3 μg pellet.

Figure 2A shows that serum IGF-I decreased to control levels in $Tx_5+RP_{1·5}$ and to levels below those of controls in Tx_5+RP_3 in parallel with the decrease in insulin, while liver mRNA expression of IGF-I remained increased in $Tx_5+RP_{1·5}$ but returned to control values in Tx_5+RP_3, in which insulin was more reduced and the euthyroid condition was attained. The increase in serum IGF-II induced by Tx was already reduced below control values in $Tx_5+RP_{1·5}$. In agreement, a significant reduction of the Tx-induced rise in liver mRNA expression of IGF-II was observed after T_4 treatment in $Tx_5+RP_{1·5}$ and Tx_5+RP_3 (Fig. 2B). These results indicate that T_4 treatment by pellet implantation leads to a more complete recovery of IGF-I than after T_4 treatment by injection, as occurs with all parameters depicted in Table 1.

Table 2 shows the increased plasma TSH and the decreased body weight, serum T_3, T_4, GH and insulin and pituitary GH content after Tx in adult populations. Although increases in body weight, plasma GH and insulin and pituitary GH content were observed after the three treatments, the values reached in $Tx_{72}+RP_{10}$ were closer to those of controls than those of $Tx_{72}+RP_5$, and the latter were better than those of Tx_{72}+R in which T_4 was administered by injection. $Tx_{72}+RP_5$ and $Tx_{72}+RP_{10}$ showed serum T_3 and T_4 levels approaching or similar to those of controls, while plasma TSH levels only decreased to control values in $Tx_{72}+RP_{10}$. Plasma GH did not reach control values in any group and pituitary GH content only recovered in $Tx_{72}+RP_{10}$, the group in which plasma TSH decreased to control values. Finally, only T_4 administered by pellet, $Tx_{72}+RP_5$ and $Tx_{72}+RP_{10}$, led to recovered plasma insulin levels, while blood glucose was not altered in Tx rats compared with controls but was increased in Tx_{72}+R and $Tx_{72}+RP_5$.

Figure 3 shows that serum levels and liver mRNA expression of IGF-I increased in Tx adult rats after all T_4 treatments but reached values close to those of controls only in $Tx_{72}+RP_{10}$, when the euthyroid condition was attained, as shown by the restored plasma TSH levels. These results show that the best recovery of circulating thyroid hormones and IGF-I to steady-state levels after T_4 administration was obtained in the $Tx_{72}+RP_{10}$ group and that, overall, pellet implantation was a better treatment than injection.

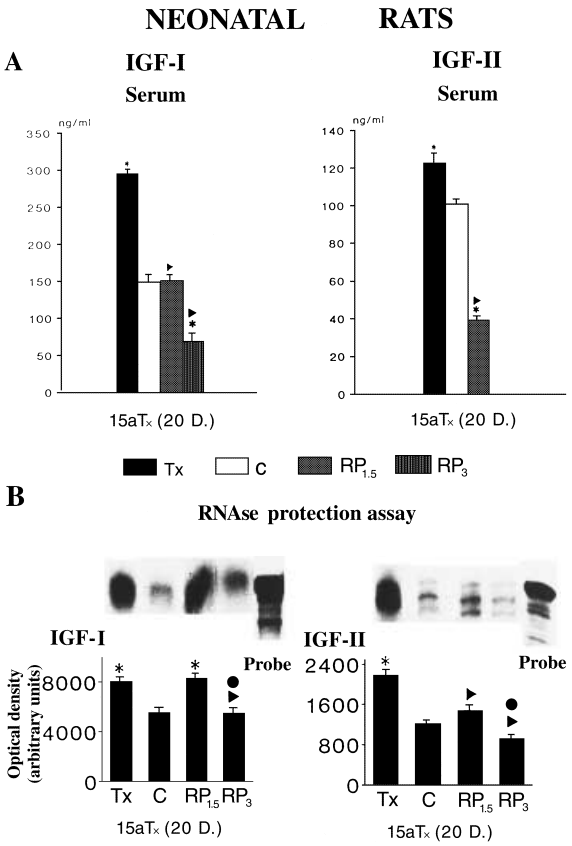

Figure 2 (A) Serum IGF-I by RIA and IGF-II by radioreceptor assay in neonatal thyroidectomized (Tx), control (C) and Tx treated with T_4 (1·5 μg/100 g b.w. ($RP_{1·5}$) or 3 μg/100 g b.w. per day (RP_3)) by pellet implantation. Plasma from 3–4 different rats was pooled and eight different pools per group 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 ± S.D. *P<0·05 relative to control rats; ▶P<0·05 relative to Tx rats; ●P<0·05 relative to $RP_{1·5}$ rats.

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

Table 2 Body weight, T_3, T_4, TSH, insulin and GH plasma levels and GH pituitary content in thyroidectomized (Tx) adult rats treated with T_4 (1·75 μg/100 g b.w.) by i.p. injection (Tx_{72}+R) or s.c. pellet with the same doses for 5 days ($Tx_{72}+RP_5$) or for 10 days ($Tx_{72}+RP_{10}$). Adult rats were killed at 87 or 92 days of age. Results are means ± S.D., n=8–10

	Body weight (g)	Serum T_3 (ng/dl)	Serum T_4 (μg/dl)	Plasma TSH (ng/ml)	Plasma GH (ng/ml)	Pituitary GH (μg/mg)	Blood glucose (mg/100 ml)	Plasma insulin (μU/ml)
Tx_{72}	167·20 ± 3·50[a]	7·90 ± 0·30[a]	0·40 ± 0·08[a]	396 ± 68·3[a]	14·35 ± 1·32[a]	10·77 ± 0·72	130·63 ± 1·71[a]	48·72 ± 2·62[a]
Controls	194·00 ± 5·00	94·53 ± 4·46	5·30 ± 0·70	8·6 ± 1·4[b,c,d]	96·93 ± 4·46	33·12 ± 1·16	133·05 ± 1·90	66·95 ± 7·55
Tx_{72}+R	171·95 ± 4·05[a]	26·07 ± 2·59[a,b]	1·96 ± 0·54[a,b]	340·1 ± 58·2[a]	59·67 ± 5·73[a,b]	13·49 ± 0·93[a]	140·21 ± 4·77[b]	50·61 ± 2·59[a]
$Tx_{72}+RP_5$	188·78 ± 5·48[b,c]	99·45 ± 5·77[b,c]	6·77 ± 0·12[b,c]	339·8 ± 57·3[a]	60·77 ± 3·46[a,b]	26·32 ± 2·64[a,b,c]	141·45 ± 3·26[b]	55·02 ± 5·80[b]
$Tx_{72}+RP_{10}$	189·63 ± 4·38[b,c]	74·02 ± 2·69[b,c]	5·89 ± 1·04[b,c]	6·8 ± 0·2[b,c,d]	70·61 ± 6·11[a,b]	33·89 ± 2·58[b,c,d]	133·15 ± 3·01	64·43 ± 4·29[b,c]

[a]P<0·05 relative to C rats; [b]P<0·05 relative to Tx rats; [c]P<0·05 relative to Tx+R rats; [d]P<0·05 relative to $Tx+RP_5$ rats.

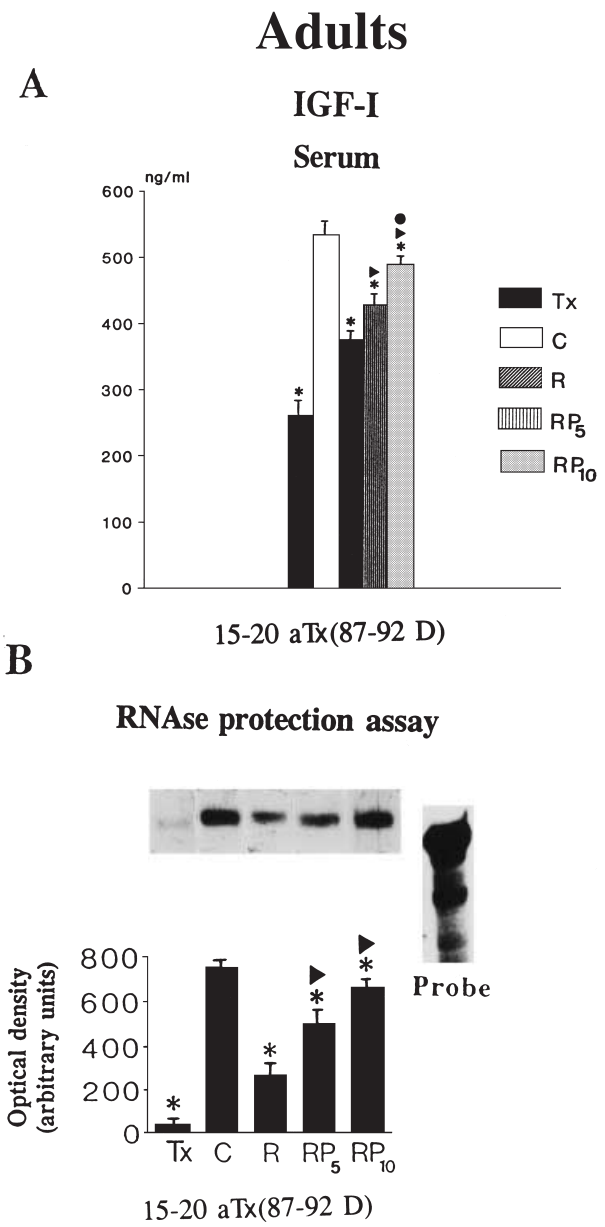

Figure 3 (A) Serum IGF-I in adult rats thyroidectomized (Tx), control (C) and Tx treated with T_4 (1·75 µg/100 g b.w. each day) by i.p. injection (R) or s.c. pellet for 5 (RP_5) or 10 days (RP_{10}). All groups were killed 15 days after Tx on day 87 except for RP_{10} group which was killed at 92 days 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; ●P<0·05 relative to R rats.

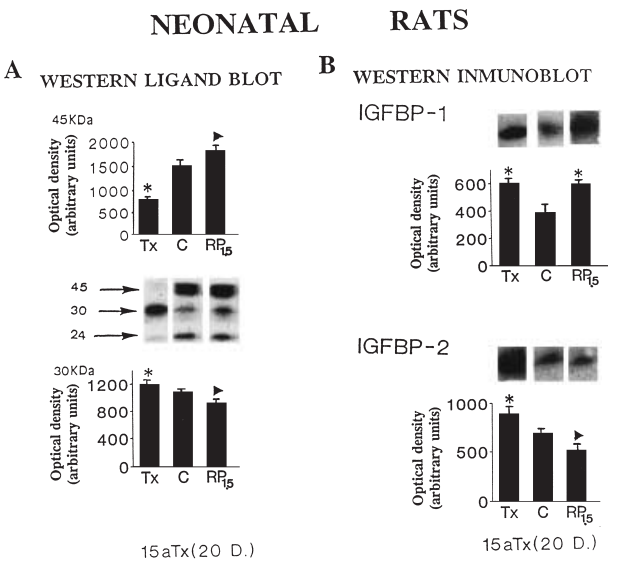

Figure 4 (A) Western ligand blot of neonatal rats thyroidectomized (Tx), control (C) and Tx treated with T_4 by pellet implantation ($RP_{1·5}$) (1·5 µg/100 g b.w.) for 5 days. (B) Western immunoblot of IGFBP-1 and IGFBP-2 in the same populations, to evaluate the IGFBP-1 and -2 ratio in the 30 kDa complex 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 rats 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.

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 T_3 (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).

Western ligand blot and immunoblot of serum IGFBP levels and liver mRNA expression of IGFBPs in thyroidectomized neonatal and adult rats treated with T_4 (Figs 4, 5 and 6)

Western ligand blot showed that the 45 kDa band of IGFBPs (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+$RP_{1·5}$; therefore, a greater dose of T_4 was not assayed. Parallel to

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 $Tx_5+RP_{1·5}$ and Tx_5+RP_3, but IGFBP-2 transcripts reached levels below those of controls in $Tx_5+RP_{1·5}$ and were even lower in Tx_5+RP_3. The results obtained after T_4 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 T_4 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 T_4 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 T_4 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 $Tx_{72}+RP_{10}$.

In adult rats, all T_4 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 Tx_{72}+R (Fig. 5). IGFBP-2 decreased below the levels of controls in Tx_{72}+R and $Tx_{72}+RP_5$, and IGFBP-3 increased to values above those of controls in $Tx_{72}+RP_5$ and $Tx_{72}+RP_{10}$. 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 Tx_{72}+R, a complete recovery of the serum levels of IGFBPs was obtained only in $Tx_{72}+RP_{10}$, in which the euthyroid condition was attained. These results confirm that pellet implantation for 10 days ($Tx_{72}+RP_{10}$) is the most suitable T_4 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 T_4 administration (Figs 5 and 6).

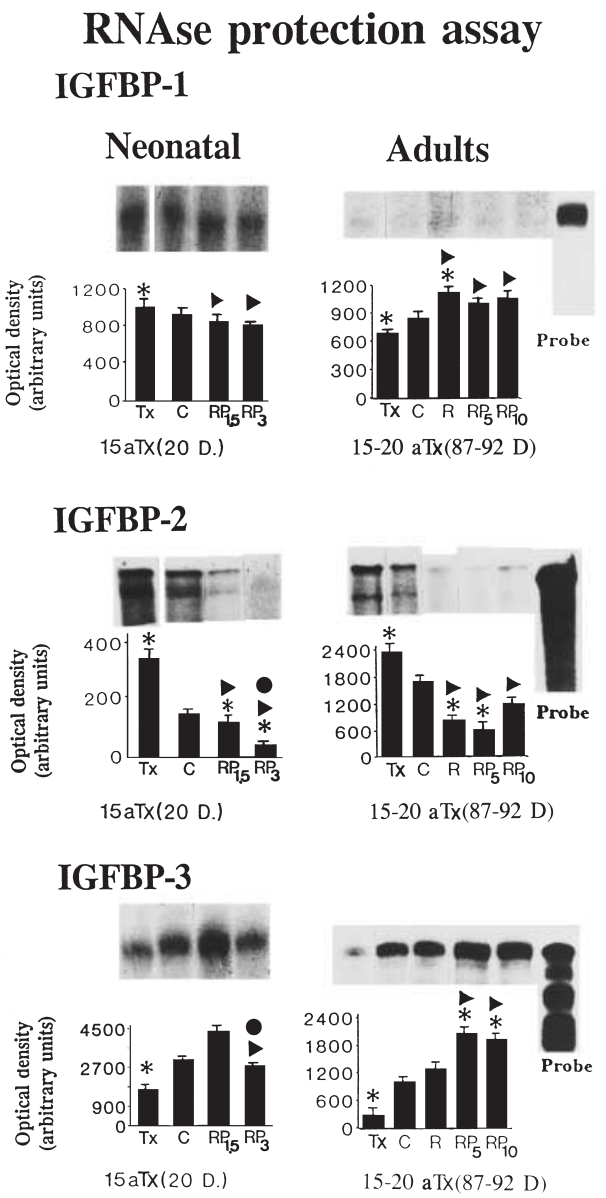

Figure 5 Liver mRNA expression of IGFBP-1, IGFBP-2 and IGFBP-3 in neonatal and adult rats thyroidectomized (Tx), control (C), and neonatal rats treated with T_4 by pellet ($RP_{1·5}$: 1·5 μg/100 g b.w. per day or RP_3: 3 μg/100 g b.w. per day) for 5 days, and adult rats treated with pellet (1·75 μg/100 g b.w. per day) for 5 (RP_5) or 10 days (RP_{10}). All animals were killed at the indicated times (20 D, 87–92 D) (see also Fig. 1). A representative experiment of bands of RNase protection assay (RPA) is depicted in the figure. Densitometric quantification of all bands from the same group is also shown as arbitrary units. For the neonatal group, four different samples per group were assayed in two separate RPAs with a total of eight animals analyzed. For the adult group, three different samples per group were assayed in two 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; ●P<0·05 relative to $RP_{1·5}$ or R rats.

Discussion

Influence of T_4 dose and method of administration

Administration of T_4 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).

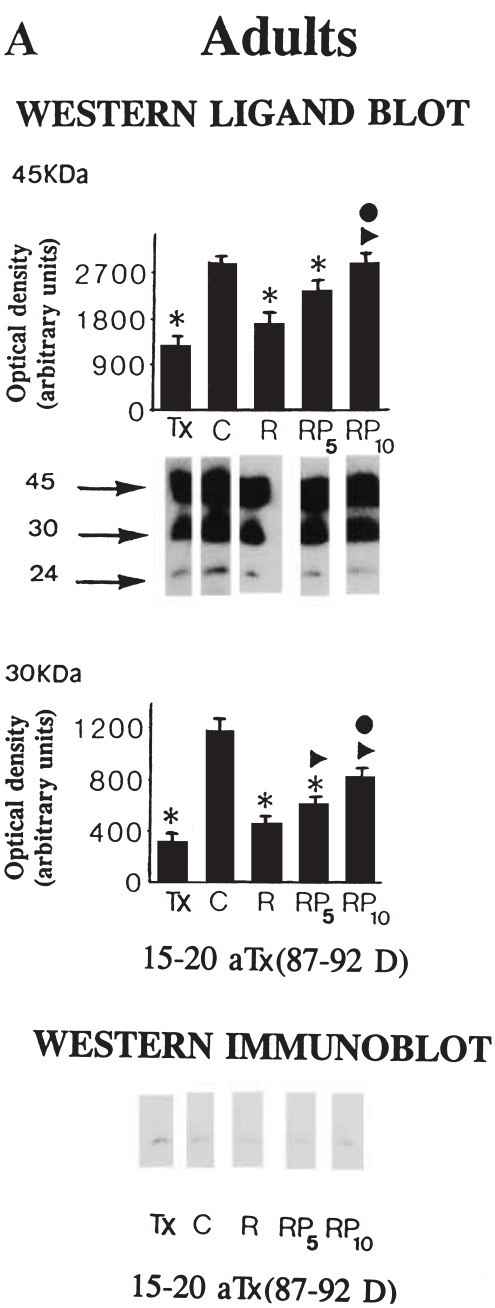

Figure 6 (A) Western ligand blot (WLB) of adult rats thyroidectomized (Tx), control (C) and Tx treated with T_4 (1·75 μg/100 g b.w.) by i.p. injection (R) or by pellet implantation for 5 (RP_5) or 10 days (RP_{10}) (see Fig. 1). (B) Western immunoblot specific for IGFBP-2 in the same populations to quantitate 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.

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 T_4 pellet implantation, when the euthyroid condition was established. In this study, T_4 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 T_4 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 T_4 compared with controls (Fig. 2), as well as in adult rats from Tx_{72}+R to Tx_{72}+RP_{10} (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 (Tx_{72}+RP_{10}). 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 T_4, 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 T_4 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

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 T_4

The utilization of the smallest dose of T_4 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 T_4 (3 μg). External doses of T_4 (1·5 or 3 μg) decrease IGFs in neonates in parallel to the dose–response decrease in insulin and despite the T_4-induced increase in GH (Table 1). The latter results from a well known direct effect of thyroid hormones at the pituitary, and T_4 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 T_4 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 multivariance 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 T_4. The results also show that, although the small dose of T_4 administered by pellet ($RP_{1·5}$) 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 T_4 doses than IGF-I during the neonatal period.

Elevated insulin observed in Tx neonatal rats decreased after T_4 replacement but did not reach control levels, while no changes in serum IGFBP-1 were observed in Tx_5+$RP_{1·5}$ 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 T_4-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 T_4 treatment of Tx neonatal rats induced a dose-dependent decrease in IGFBP-2. Thus, the results obtained after T_4 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 T_4 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 T_4-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 T_4

As previously described (Ramos *et al.* 1998), a decrease in circulating thyroid hormones, GH and insulin was found in Tx adult rats. After T_4 administration, the progressive recovery of the metabolic and endocrine parameters observed in adult rats from Tx_{72}+R to TX_{72}+R_{10}, more evident than in neonates treated with increasing doses of T_4, 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 T_4 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 multivariance 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. RP_{10} stands out as the most suitable method for T_4 restoration, and a progressive recovery of all IGFBPs from R to RP_{10} adult rats was found. The parallel recovery of liver mRNA expression and serum concentrations of IGFBP-3, the most abundant IGFBP in adulthood, after T_4 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 T_4 treatment, a regulatory pattern previously reported (Jones & Clemmons 1995, Ramos *et al.* 1998). The T_4-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 T_4 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 T_4 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 (Näntö-Salonen *et al.* 1991). Moreover, Western immunoblot and RNase protection assay showed increased IGFBP-2 in Tx adult rats and its reduction after T_4 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 T_4 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 T_4 administration, show a differential mechanism of recovery of the IGFs/IGFBPs system in Tx rats treated with T_4 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 IGFs decrease after T_4 administration to Tx neonatal rats despite the rise in GH, showing that in immature stages insulin, rather than GH, regulates IGFs. Besides, the results obtained suggest a possible direct effect of thyroid hormones on the IGF regulation *in vivo* since a complete recovery of IGFs is observed only when the euthyroid condition has been ensured, although further research is needed to unravel the mechanism.

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