Tri-iodothyronine increases insulin-like growth factor binding protein-2 expression in cultured hepatocytes from hypothyroid rats

I Demori, C Bottazzi and E Fugassa

Istituto di Fisiologia Generale, Università di Genova, Corso Europa 26, I-16132 Genova, Italy
(Requests for offprints should be addressed to E Fugassa)

Abstract

Previous evidence suggests the existence of a thyroid hormone–IGF axis in the liver and changes in hepatic insulin-like growth factor binding protein (IGFBP) expression in rats with altered thyroid status have been previously reported.

The aim of this study was to check if the higher IGFBP-2 mRNA levels observed in liver of hypothyroid rats could be due to a direct effect of thyroid hormone on the IGFBP-2 gene. In our experiments, cultured hepatocytes isolated from normal and hypothyroid adult rats were used. Northern blot analysis revealed barely detectable IGFBP-2 mRNA in normal rat hepatocytes, but easily detectable signal in hypothyroid rat cells. Therefore, the effect of tri-iodothyronine (T_3) was investigated using cultured hepatocytes from hypothyroid rats as an *in vitro* model.

The IGFBP-2 message was increased in a dose-dependent manner in hepatocytes cultured for 12–24 h in the presence of T_3 . A similar increase occurred in accu-

mulation of IGFBP-2 in the culture medium, as measured by RIA. The effect of T_3 on IGFBP-2 transcript levels appeared to consist of enhanced gene transcription and was independent of ongoing protein synthesis, but it was completely abolished by the incubation of hepatocytes with insulin. The latter result confirmed the dominant role of insulin in regulating IGFBP-2 expression by cultured hepatocytes.

In vivo experiments confirmed an increase in hepatic IGFBP-2 mRNA and serum IGFBP-2 levels in hypothyroid rats and demonstrated, in addition, a significant increase in these measures in T_3 -treated rats.

Taken together, our *in vitro* and *in vivo* results support a role for a thyroid hormone–IGF axis in the liver and suggest that other factors, such as insulin, interact *in vivo* with thyroid hormone in regulating hepatic IGFBP-2 expression.

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Introduction

The insulin-like growth factors (IGF-I and -II) are polypeptide hormones, similar in structure to proinsulin, which play important roles in cell growth and differentiation. The IGFs are found, in the circulation and in the extracellular fluids, bound to specific high-affinity binding proteins (IGFBP-1 to -6) that are unrelated to either type of the IGF-receptors. The IGFBPs are potent modulators of IGF actions on target cells and have been reported both to inhibit and to potentiate IGF activity (Froesch *et al.* 1985, Rechler 1993, Jones & Clemmons 1995).

The components of the IGF/IGFBP system are produced by different cells and tissues, the liver being one of the major sites of synthesis. The levels of IGFBPs in serum and IGFBP mRNAs in liver are highly regulated by metabolic and hormonal factors as well as by the developmental age.

In adult rat serum, most of the IGFs circulate as a 150-kDa ternary complex that consists of IGF-I, IGFBP-3

and an acid-labile subunit (ALS). In contrast, in fetal rat serum there are high levels of IGF-II and the most abundant binding protein is IGFBP-2 (Lund *et al.* 1986, Donovan *et al.* 1989, Ooi *et al.* 1990, Albiston & Herington 1992).

In the rat, IGFBP-2 mRNA has been detected in several fetal tissues, the highest levels being in the liver (Brown *et al.* 1989). Although message levels are barely detectable in adult liver, relatively high levels persist in adult brain, testes, ovaries and kidney (Margot *et al.* 1989).

The factor(s) responsible for the regulation of IGFBP-2 mRNA within rat tissues are not well defined. Hepatic IGFBP-2 transcript is increased by hormonal and metabolic manipulation of adult animals such as fasting, hypophysectomy and diabetes (Böni-Schnetzler *et al.* 1989, Margot *et al.* 1989, Orlowski *et al.* 1990, Ooi *et al.* 1990, Tseng *et al.* 1992). Interestingly, the increase after hypophysectomy is not normalized by growth hormone (GH) treatment (Margot *et al.* 1989, Ooi *et al.* 1990), suggesting a possible involvement of other hormones regulated by

pituitary secretion. A candidate for this role could be thyroid hormone: it has been demonstrated that thyroid hormone withdrawal increases the expression of IGFBP-2, in both developing and adult rat liver (Näntö-Salonen *et al.* 1991, Rodríguez-Arno *et al.* 1994). GH replacement in hypothyroid animals did not correct the increase in IGFBP-2 message, suggesting a possible direct regulation of this gene by thyroid hormone (Näntö-Salonen *et al.* 1993).

We have recently demonstrated a tri-iodothyronine (T_3)-induced increase in IGFBP-4 expression in a rat hepatocyte model, in total agreement with the *in vivo* effects of thyroid hormone withdrawal from or administration to the rats (Demori *et al.* 1997). In the same experiments, we were unable to obtain evidence of an effect of T_3 on IGFBP-2 expression, because of the barely detectable level of IGFBP-2 message in cultured hepatocytes from adult rats.

In the present study, we used the *in vitro* model of primary hepatocytes isolated from hypothyroid rats, which express high levels of IGFBP-2 mRNA, enabling us to demonstrate the direct regulation of IGFBP-2 expression by T_3 .

Materials and Methods

Animals

Adult male Wistar rats were rendered hypothyroid by the addition of 0.05% propylthiouracil (PTU) or 0.05% methimazole (MMI) to the drinking water from the 6th to the 15th week of age. Age-matched rats receiving regular drinking water served as controls. During the last week before being killed, groups of both hypothyroid and control rats were treated daily with i.p. injections of T_3 (15 µg/100 g body weight).

Animals were killed by cervical dislocation. Blood was collected, allowed to clot and the serum stored at -80°C . Serum T_3 and thyroxine (T_4) were evaluated by an enhanced chemiluminescent (ECL) enzyme immunoassay (DPC, Los Angeles, CA, USA). The limit of detection of the assay was 0.35 ng/ml for T_3 and 4 ng/ml for T_4 .

Immediately after death of the rat, the liver was promptly removed, quickly frozen in liquid nitrogen and stored at -80°C until required for use.

Hepatocyte primary cultures

Male Wistar rats rendered hypothyroid by PTU administration were used to isolate hepatocytes by a standardized collagenase (type I, Sigma Chemical Co., St Louis, MO, USA) perfusion technique as previously described (Fugassa *et al.* 1983). Cells (viability $\geq 85\%$ by trypan blue exclusion) were resuspended at a concentration of 5×10^5 cells/ml in serum-free Dulbecco's modified Eagle's

medium (DMEM) supplemented with essential and non-essential amino acids, insulin (0.1 U/ml) and dexamethasone (10^{-6} M). Next, 8 ml of this cell suspension were plated on collagen (type III, Sigma)-coated 100-mm plastic dishes (Costar, Cambridge, MA, USA). After 90 min, medium with unattached cells was aspirated and monolayers were exposed to DMEM containing 0.25% BSA (fraction V, cell culture tested, Sigma). Specific experiments were begun on cultures that had been incubated for 24 h of adaptation to the *in vitro* environment (time 0). T_3 (Sigma) was added to the culture medium at indicated concentrations. Insulin (Actrapid HM, Novo Nordisk, Bagsvaerd, Denmark) was added to the culture medium at the concentration of 10^{-8} M. The protein synthesis inhibitors, cycloheximide (0.1 µg/ml; Sigma) and anisomycin (2.65 µg/ml; Sigma), were added to the cultures 1 h before the addition of T_3 . The transcription inhibitor, actinomycin D (Sigma), was used at a concentration of 5 µg/ml. PTU (Sigma) was used at a dose of 5.8×10^{-9} M (Cook *et al.* 1983).

Cell viability was not affected by the duration of the culture or by the addition of drugs to the medium, as checked by trypan blue exclusion test.

At the indicated times, culture medium was removed; monolayers were quickly washed twice with ice-cold sterile PBS, immediately frozen in liquid nitrogen and stored at -80°C until used for RNA extraction.

RNA isolation

Total RNA was prepared using acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski & Sacchi 1987). RNA was quantified by measuring absorbance at 260 nm. Samples were stored at -80°C until required for use.

cDNA probes

The rat IGFBP-2 (Brown *et al.* 1989) cDNA probe was kindly provided by Dr S Shimasaki, Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, USA. The human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe was purchased from Clontech Laboratories Inc., Palo Alto, CA, USA. The rat 18S rDNA probe (BamHI-HindIII fragment, Chan *et al.* 1984) was generously provided by Dr I G Wool, Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL, USA. The probes were labelled to high specific radioactivity by multiprime DNA labelling systems (Amersham International plc, Amersham, Bucks, UK) using [^{32}P]dCTP (NEN Du Pont, Du Pont De Nemours GmbH, Bad Homburg, Germany; specific radioactivity 3000 Ci/mmol).

Northern blot analysis

Total RNA (20 µg) was fractionated by loading on 1% agarose gel containing 2.2 M formaldehyde in 20 mM

MOPS (pH 7.0), 5 mM sodium acetate and 1 mM di-sodium EDTA. RNA was stained with ethidium bromide to check the integrity of the samples and ensure equal loading in each lane. After transfer of the samples to nylon membrane by capillary blotting, prehybridization was performed for 2 h at 42 °C in a solution containing $5 \times$ SSPE ($1 \times$ SSPE = 0.15 M NaCl–0.01 M NaH_2PO_4 –1 mM EDTA), 50% formamide, $5 \times$ Denhardt's solution, 0.5% SDS and 100 µg/ml denatured salmon sperm DNA (Sambrook *et al.* 1989). Hybridization was carried out for 20 h at 42 °C in fresh buffer containing the ^{32}P -labelled probe. After hybridization, the filters were washed sequentially in $2 \times$ SSPE, 0.1% SDS, $1 \times$ SSPE, 0.1% SDS and $0.1 \times$ SSPE, 0.1% SDS at 42 °C, each wash lasting for 20 min. Filters were then exposed to Amersham Hyperfilm-MP films at -80 °C using intensifying screens.

To ensure even loading, the membranes were stripped and rehybridized with either a human G3PDH cDNA probe or a rat 18S rDNA probe.

The signal intensity of the autoradiograms was quantified densitometrically using the Scion ImagePC software package. Arbitrary densitometric units were normalized to G3PDH or 18S and expressed as a percentage of the control values.

IGFBP-2 determination in serum and conditioned medium

IGFBP-2 concentrations in rat serum and hepatocyte conditioned medium were measured by RIA (DSL, Webster, Texas, USA), with a limit of detection of 0.5 ng/ml.

Statistical analyses

Data are presented as the mean $\pm$ S.E.M. Statistical significance was analysed using ANOVA with the multiple group comparison test of Scheffé. Differences were regarded as significant when $P \leq 0.05$.

Results

IGFBP-2 mRNA levels in cultured hepatocytes

Hepatocytes were incubated for 24 h to allow adaptation to the *in vitro* environment, then transferred into fresh DMEM supplemented as described under Materials and Methods. At different times, total RNA was extracted and analysed for the presence of IGFBP-2 mRNA by Northern blot hybridization. As shown in Fig. 1, Northern blot analysis revealed barely detectable IGFBP-2 mRNA in normal rat hepatocytes, according to Arany *et al.* (1993). In contrast, when cells were prepared from PTU-treated hypothyroid rats, the IGFBP-2 message was readily detected. No direct effect of PTU on IGFBP-2 mRNA

Figure 1 IGFBP-2 mRNA levels in cultured rat hepatocytes. Representative autoradiograms of Northern blots showing 1.5 kb IGFBP-2 mRNA, 1.3 kb G3PDH mRNA and 1.9 kb 18S rRNA. Lanes: 1, adult rat liver; 2, hypothyroid rat liver; 3, adult rat hepatocytes cultured for 24 h; 4, adult rat hepatocytes cultured for 24 h in the presence of 10^{-6} M T_3 ; 5, hypothyroid rat hepatocytes cultured for 24 h; 6, hypothyroid rat hepatocytes cultured for 24 h in the presence of 10^{-6} M T_3 ; 7, adult rat hepatocytes cultured for 24 h; 8, adult rat hepatocytes cultured for 24 h in the presence of 5.8×10^{-9} M PTU.

levels was seen when the drug was added to the culture medium of normal hepatocytes. Therefore, the effect of T_3 was investigated using cultured hepatocytes isolated from hypothyroid rats.

Effect of T_3 on IGFBP-2 expression in cultured hepatocytes

Figure 2 shows the characteristic 1.5 kb IGFBP-2 message expressed by rat hepatocytes cultured for up to 24 h after the first 24 h of adaptation to the culture conditions. It appears that IGFBP-2 mRNA levels increased with the time in culture. When 10^{-6} M T_3 was present in the culture medium for 12–24 h, the abundance of IGFBP-2 mRNA was significantly enhanced ($P < 0.001$).

IGFBP-2 concentration in conditioned media of hepatocytes cultured for 12 h was 11.5 ± 1.8 ng/ml, and this value was increased in 10^{-6} M T_3 -treated cells (19.7 ± 1.7 ng/ml, $P \leq 0.03$), consistent with what was observed at the mRNA level.

Figure 3 shows the levels of IGFBP-2 mRNA after the addition of T_3 to the culture medium to final concentrations ranging from 10^{-10} to 10^{-6} M. After 12 h of exposure, the maximal increase in IGFBP-2 mRNA levels was elicited by 10^{-6} – 10^{-8} M T_3 , whereas 10^{-9} and 10^{-10} M were ineffective.

Effect of cycloheximide and anisomycin on T_3 -induced IGFBP-2 mRNA levels in cultured hepatocytes

Hepatocytes were cultured for 12 h in the presence of either cycloheximide (0.1 µg/ml) or anisomycin (2.65 µg/ml), which both inhibit *de novo* protein synthesis. The inhibitors were used at doses that did not decrease cell viability over the time of incubation, but were able to inhibit protein synthesis in cultured hepatocytes by 90–97% (Barreca *et al.* 1992, Messina 1991).

As shown in Fig. 4, the presence of cycloheximide or anisomycin in the culture medium resulted in increased

Figure 2 Time course of the T_3 effect on IGFBP-2 mRNA levels in hypothyroid rat hepatocytes cultured in the absence or presence of 10^{-6} M T_3 . (A) Representative autoradiograms of Northern blots showing 1.5 kb IGFBP-2 and 1.3 kb G3PDH mRNAs. (B) Densitometric quantification of Northern blots shown in (A) after normalization to G3PDH. Hatched bars, with T_3 ; open bars, without T_3 . Results are expressed as percentage of the control at time 0 (mean $\pm$ S.E.M.; $n=3$). * $P<0.02$ compared with time 0; *** $P<0.001$ compared with respective controls.

overall amount of IGFBP-2 mRNA, but did not affect the effect of T_3 on the specific transcript.

Effect of actinomycin D on T_3 -induced IGFBP-2 mRNA levels in cultured hepatocytes

To check the effect of T_3 on IGFBP-2 mRNA stability, hepatocytes were cultured for 12 h in the absence or presence of T_3 to achieve the induction of IGFBP-2 mRNA, and then exposed to actinomycin D (5 μ g/ml), a potent inhibitor of transcription, in order to evaluate IGFBP-2 mRNA decay.

As shown in Fig. 5, both control and T_3 -treated hepatocytes showed stable IGFBP-2 mRNA levels, even after 12 h of incubation with actinomycin D. Owing to

Figure 3 Dose-dependence of the effect of T_3 on IGFBP-2 mRNA levels in hypothyroid rat hepatocytes cultured for 12 h in the absence or presence of different doses of T_3 . (A) Representative autoradiograms of Northern blots showing 1.5 kb IGFBP-2 and 1.3 kb G3PDH mRNAs. (B) Densitometric quantification of Northern blots after normalization to G3PDH. Results are expressed as percentage of control (open bar) (mean $\pm$ S.E.M.; $n=3$). * $P<0.001$ compared with control.

the lack of decay of IGFBP-2 mRNA, it was impossible to evaluate its half-life and consequently the effect of T_3 on IGFBP-2 mRNA stability.

As shown in Fig. 6, the presence of actinomycin D along with T_3 in the culture medium of hepatocytes cultured for 12 h prevented the T_3 -induced increase in IGFBP-2 mRNA levels, suggesting a transcriptional regulation of IGFBP-2 gene by T_3 .

Effect of insulin on T_3 -induced IGFBP-2 mRNA levels in cultured hepatocytes

It has been reported that insulin decreases IGFBP-2 mRNA levels in cultured hepatocytes (Böni-Schnetzler

Figure 4 Effects of cycloheximide (Cex) (0.1 µg/ml) and anisomycin (Aniso) (2.65 µg/ml) on IGFBP-2 mRNA levels in hypothyroid rat hepatocytes cultured for 12 h in the presence (+) or absence (-) of 10⁻⁶ M T₃. (A) Representative autoradiograms of Northern blots showing 1.5 kb IGFBP-2 and 1.3 kb G3PDH mRNAs. (B) Densitometric quantification of Northern blots after normalization to G3PDH. Results are expressed as percentage of control (□) (mean ± S.E.M.; n=3). *P<0.03 compared with control; †P<0.05 compared with Cex- or Aniso-treated.

et al. 1990). We tested the effect of the pancreatic hormone on basal and T₃-induced IGFBP-2 expression in our culture system. As can be seen in Fig. 7, the presence of 10⁻⁸ M insulin in the culture medium for 12 h resulted in decreased levels of IGFBP-2 message. When insulin was present in the culture medium of hepatocytes together with T₃, the stimulatory effect of the latter hormone was completely abolished.

Effect of T₃ administration on IGFBP-2 expression in rat liver

The finding that hepatic IGFBP-2 expression is enhanced *in vivo* by thyroid hormone withdrawal and *in vitro* by addition of T₃ to the culture medium of hepatocytes from hypothyroid rats induced us to verify the effects of T₃ administration to both hypothyroid and euthyroid rats.

Groups of control and hypothyroid rats were injected daily with T₃ (15 µg/100 g body weight) during the last week before death.

Serum T₃ and T₄ levels of the animals are reported in Table 1. PTU treatment always resulted in lower serum

concentrations of thyroid hormone with respect to controls. The same results were obtained when the rats were treated with MMI (not shown). T₃ treatment induced high serum T₃ concentrations, associated with low serum concentration of T₄.

Figure 8 shows that PTU-induced hypothyroidism was associated with enhanced expression of hepatic IGFBP-2 mRNA. T₃ treatment decreased hepatic IGFBP-2 mRNA to about 50% of the levels of hypothyroid rat liver. When T₃ was given to control rats, the hepatic IGFBP-2 signal was faint but reproducible and significantly greater compared with the undetectable signal in control livers. The changes in IGFBP-2 levels in the serum of the same rats paralleled those of the hepatic mRNA in all conditions tested (Table 2).

As shown in Fig. 9, the amount of IGFBP-2 mRNA was increased in the liver of MMI-treated hypothyroid rats compared with controls. The abundance of the IGFBP-2 transcript in the liver of T₃-replaced rats was lower than that in hypothyroid animals, but higher with respect to controls. A similar pattern was observed for serum IGFBP-2 levels (not shown).

Discussion

In the rat, the IGFBPs are produced by different tissues and cell types under a complex and specific regulation by hormonal, nutritional and developmental factors. During fetal and early postnatal life, the liver produces high levels of IGFBP-2, but within the first 3 weeks after birth hepatic IGFBP-2 production strongly decreases to the barely detectable levels typical of the adult rat. It has been reported that congenital hypothyroidism delays the decline of hepatic IGFBP-2 expression after birth (Näntö-Salonen *et al.* 1991), and an increase in hepatic IGFBP-2 mRNA has also been observed in rats rendered hypothyroid in adulthood (Rodriguez-Arnan *et al.* 1994). Therefore, thyroid hormone appears to play a role in regulating hepatic IGFBP-2 production in newborn and adult rats. However, our previous attempts to demonstrate a direct effect of T₃ on IGFBP-2 expression were ineffective (Demori *et al.* 1997).

In the present study, we have analysed the effect of T₃ on hepatic IGFBP-2 expression using cultured hepatocytes isolated from hypothyroid adult rats, in which the IGFBP-2 message is easily detectable and the sensitivity to exogenous T₃ may be greater than that of cells from euthyroid animals (Mariash & Oppenheimer 1983).

The typical 1.5 kb IGFBP-2 mRNA was clearly expressed by hepatocytes from hypothyroid rats cultured for up to 24 h. The addition of T₃ to the hepatocyte culture medium increased the abundance of IGFBP-2 message, to about 300% with respect to control cells. The stimulation was highly significant after 12–24 h of incubation with T₃ in doses ranging from 10⁻⁸ to 10⁻⁶ M.

Figure 5 IGFBP-2 mRNA stability in hypothyroid rat hepatocytes cultured for 12 h (time 0) in the absence (–) or presence (+) of 10^{-6} M T_3 and then exposed to 5 µg/ml actinomycin D (ActD) for different durations of time without a change of medium. (A) Representative autoradiogram of Northern blots showing 1.5 kb IGFBP-2 and 1.3 kb G3PDH mRNAs. (B) Densitometric quantification of Northern blots after normalization to G3PDH. + T_3 , ●; – T_3 , ○. Results are expressed as percentage of control (mean ± S.E.M.; $n=3$).

IGFBP-2 peptide production also was stimulated in T_3 -treated cells.

The effect of T_3 on IGFBP-2 mRNA appeared to be independent of ongoing protein synthesis, as it was not abolished by the protein synthesis inhibitors, cycloheximide or anisomycin. The finding that the overall amount of IGFBP-2 transcript was greater in cells treated with cycloheximide or anisomycin could suggest the existence of a labile repressor protein that disappears when protein synthesis is blocked. However, direct evidence for the existence of such a protein is lacking.

The increase in IGFBP-2 mRNA steady state levels in T_3 -treated cells could be due to enhanced transcription of the gene, greater stability of the transcript, or both. In order to clarify these points, we tested the effect of

the transcription inhibitor, actinomycin D, on IGFBP-2 mRNA abundance. Our results suggest a transcriptional regulation of the IGFBP-2 gene by T_3 : when hepatocytes were treated with the hormone along with actinomycin D to block gene transcription, the stimulation of IGFBP-2 expression was abolished. Moreover, when actinomycin D was used in order to evaluate the IGFBP-2 mRNA half-life, the message showed stable levels both in T_3 -treated and in control cells, and no decay of the signal was apparent, so that a stabilizing effect of T_3 on IGFBP-2 mRNA seems unlikely.

The presence of a thyroid hormone response element in the promoter region of the rat IGFBP-2 gene has not been documented, but a specific stimulation of IGFBP-2 expression by T_3 in osteoblasts has been observed (Schmid

Figure 6 Effect of T_3 on IGFBP-2 mRNA levels in hypothyroid rat hepatocytes cultured for 12 h with (+) or without (-) 10^{-6} M T_3 in the presence (+) or absence (-) of actinomycin D (ActD) (5 μ g/ml). (A) Representative autoradiogram of Northern blots showing 1.5 kb IGFBP-2 and 1.3 kb G3PDH mRNAs. (B) Densitometric quantification of Northern blots after normalization to G3PDH. Results are expressed as percentage of control (□) (mean $\pm$ S.E.M.; $n=3$). * $P<0.001$ compared with control.

et al. 1992a). Other hormonal factors, also, such as retinoic acid and dexamethasone, which act via nuclear receptors belonging to the steroid/thyroid receptor superfamily, have been reported to regulate IGFBP-2 expression in different cells (Schmid *et al.* 1992b, Ernst & White 1996). However, the dominant regulator of IGFBP-2 mRNA levels in hepatocytes is considered to be insulin. When added to primary cultures of liver cells, insulin strongly decreases IGFBP-2 mRNA levels (Böni-Schnetzler *et al.* 1990). This result was also confirmed in our hepatocyte

Figure 7 Effect of insulin (I) (10^{-8} M) on IGFBP-2 mRNA levels in hypothyroid rat hepatocytes cultured for 12 h in the absence or presence of 10^{-6} M T_3 . (A) Representative autoradiograms of Northern blots showing 1.5 kb IGFBP-2 and 1.3 kb G3PDH mRNAs. (B) Densitometric quantification of Northern blots after normalization to G3PDH. Results are expressed as percentage of control (C) (mean $\pm$ S.E.M.; $n=3$). * $P<0.003$, † $P<0.05$ compared with control.

cultures from hypothyroid rats. We have demonstrated, in addition, that the inhibitory effect of insulin is dominant over the action of T_3 : when both hormones are present in hepatocyte culture medium, the stimulatory effect of T_3 on IGFBP-2 expression is suppressed.

The finding that the abundance of IGFBP-2 mRNA is high in hepatocytes from hypothyroid rats and that T_3 directly increases it even more indicates that the increase in hepatic IGFBP-2 transcript levels observed in hypothyroidism is not related directly to decreased thyroid hormone levels, but to other possible alterations associated with hypothyroidism. With our *in vivo* experiments, we

Table 1 Serum T₃ and T₄ concentrations in control, hypothyroid and T₃-treated rats. For details of treatments see Materials and Methods. Values are means ± S.E.M. (n=4)

Treatment	Serum T ₃ (ng/ml)	Serum T ₄ (ng/ml)
Control	0.53 ± 0.03	52.56 ± 1.33
PTU	<0.35***	<4
PTU+T ₃	4.40 ± 0.98†	<4
T ₃	2.03 ± 0.49*	<4

†P<0.02, *P<0.04, ***P<0.001 compared with control.

have demonstrated that T₃ treatment of hypothyroid rats decreases hepatic IGFBP-2 transcript and serum IGFBP-2 levels to about 50% of those in hypothyroid rats. Moreover, when T₃ was given to control animals, a modest but reproducible signal was detectable in the liver and a significant increase in IGFBP-2 concentration was measured in serum. Only the latter result is in agreement with our observations *in vitro*, demonstrating a direct stimulation of hepatic IGFBP-2 production by T₃.

The possibility that PTU may have a thyroid hormone-independent effect on IGFBP-2 expression seems unlikely

Table 2 Serum IGFBP-2 concentrations in control, hypothyroid and T₃-treated rats. For details of treatments see Materials and Methods. Values are means ± S.E.M. (n=4)

Treatment	Serum IGFBP-2 (ng/ml)
Control	136 ± 11.37
PTU	280 ± 16.79**
PTU+T ₃	162 ± 11.40***
T ₃	151 ± 13.59**

*P<0.04 compared with PTU; **P<0.01 compared with control.

as, when the drug was added to the culture medium of hepatocytes isolated from euthyroid animals, no direct effect on IGFBP-2 mRNA levels was apparent. Moreover,

Figure 8 Effect of PTU and T₃ administration on IGFBP-2 mRNA levels in rat liver. The animals were treated as described in Materials and Methods. (A) Representative autoradiograms of Northern blots showing 1.5 kb IGFBP-2 mRNA and 1.9 kb 18S rRNA. (B) Densitometric quantification of Northern blots after normalization to 18S. The signal in control (C) rats was undetectable and taken as 0. Values are the means ± S.E.M. (n=3). *P<0.001 compared with control; ††P<0.01 compared with PTU.

Figure 9 Effect of MMI administration and T₃ replacement on IGFBP-2 mRNA levels in rat liver. The animals were treated as described in Materials and Methods. (A) Representative autoradiograms of Northern blots showing 1.5 kb IGFBP-2 mRNA and 1.9 kb 18S rRNA. (B) Densitometric quantification of Northern blots after normalization to 18S. The signal in control (C) rats was undetectable and taken as 0. Values are the means ± S.E.M. (n=3). †P<0.05, ***P<0.001 compared with control; **P<0.01 compared with MMI.

with our *in vivo* experiments, we demonstrated that not only PTU- but also MMI-induced hypothyroidism was associated with enhanced expression of hepatic IGFBP-2. Therefore, the increase in IGFBP-2 expression may be due to secondary effects of hypothyroidism. As insulin is considered to be a dominant negative regulator of IGFBP-2 production by hepatocytes, and given the results of our *in vitro* experiments demonstrating that the inhibitory effect of insulin suppresses the stimulation of IGFBP-2 expression by T₃, we suggest that the effects observed *in vivo* could be due to an interplay between insulin and thyroid hormone. *In vivo* experiments indicate that hepatic IGFBP-2 message is preferentially increased in conditions of mild insulopenia, such as after hypophysectomy and fasting (Ooi *et al.* 1990, Tseng *et al.* 1992). Experimental hypothyroidism also represents a condition of insulin deficiency: plasma concentrations of insulin in hypothyroid rats have been reported to be reduced (Jolin *et al.* 1970, Doong *et al.* 1997) and peak plasma insulin responses to an intraperitoneal glucose load are lower in hypothyroid than in euthyroid rats (Jolin & Montes 1974). Also, in our PTU-treated rats we have confirmed a reduced tolerance to i.p. injection of glucose (not shown), similar to the findings of Jolin & Montes (1974). Even if the metabolic alterations associated with hypothyroidism are complex, the insulin deficiency could give a clue to explain, at least in part, the increase in hepatic IGFBP-2 message in PTU-treated rats. Moreover, it can be suggested that changes in nutrient intake and protein metabolism might play a role in the regulation of hepatic IGFBP-2 expression in hypothyroidism.

If not much is known about the factors that regulate hepatic IGFBP-2 expression *in vivo*, even less is understood concerning its physiological role. Mice lacking IGFBP-2 by targeted gene disruption are reported to be normal (Wood *et al.* 1993). IGFBP-2 appears to inhibit IGF actions, particularly those of IGF-II, although in certain specific cell types it can be a modest stimulator of IGF-I action (Jones & Clemmons 1995). In general, the IGFBP family provides a remarkably versatile regulatory system for the IGFs and the factors capable of modulating IGFBP expression could in turn regulate IGF actions.

In conclusion, this study has demonstrated a direct stimulation of hepatic IGFBP-2 expression by thyroid hormone although, *in vivo*, other factors, such as insulin, appear to be involved in the regulation of hepatic IGFBP-2 in different thyroid status.

Taken together with our previous data (Demori *et al.* 1997), the results presented in this paper provide further evidence that thyroid hormone interacts with the hepatic IGF system directly at the level of IGFBPs, and support a role for a thyroid hormone-IGF axis.

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