Thyroid hormone regulates tubulin expression in mammalian liver. Effects of deleting thyroid hormone receptor-α or -β

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Vallejo, Carmen G., Ana M. Seguido, Pilar S. Testillano, and María-Carmen Risueño. Thyroid hormone regulates tubulin expression in mammalian liver. Effects of deleting thyroid hormone receptor-α or -β. Am J Physiol Endocrinol Metab 289: E87–E94, 2005. First published February 15, 2005; doi:10.1152/ajpendo.00436.2004.—Microtubules are made from polymers of α/β dimers. We have observed in rat liver that, on the first day after birth, α-subunit is relatively high and β-subunit low with respect to adult values. In the hypothyroid neonate, both subunits were found to be low, therefore indicating that thyroid hormone (TH) regulates these developmental changes. TH was also found to activate tubulin expression in adult liver, especially β-subunit. To investigate the role of TH receptors (TRs) in tubulin expression, we analyzed mice lacking TRα or TRβ compared with the wild type in both normal and TH-deprived adult animals. The results suggest that, in vivo, β-tubulin protein expression in the liver is primarily under TRβ positive control. In euthyroid mice lacking TRβ, β-tubulin expression was low. However, in the corresponding hypothyroid animals, it was found increased, therefore suggesting that the unliganded TRα might also upregulate β-tubulin expression. Accordingly, TH administration to hypothyroid TRβ-deprived mice reduced their high β-tubulin expression. In parallel, the relatively high messenger level observed with these hypothyroid animals was reduced to the euthyroid level after T3 treatment. The microtubular network of the mutant livers appeared disorganized and decreased to a different extent depending on the deleted TR isoform, thus suggesting that TH regulates both tubulin expression and organization.

EXPERIMENTAL PROCEDURES

Animals and treatments. Maintenance and handling of animals were in the IIB animal facility (no. 28079-37-A), according to BOE RD 233/88 and EEC Directive 86/609. The animal procedures were revised and approved by the Consejo Superior de Investigaciones Científicas Bioethics Committee. For rat studies, adult male Wistar rats produced in our animal facilities were used. Hypothyroidism was induced by surgical thyroidectomy and the supply of 0.05% 2-mercapto-1-methylimidazole in the drinking water for ≤1 mo before the start of the experiment. In the TH-treated rats, T3 (20 μg/100 g body wt) was administered daily by intraperitoneal injections for 3 days. The normal (euthyroid) and hypothyroid animals received only vehicle injections. In the experiments with postnatal rats, to induce hypothyroidism, 0.02% 2-mercapto-1-methylimidazole and 1% sodium perchlorate were administered daily in the drinking water to the pregnant rats from day 9 of pregnancy, treatment that was continued until the animals were killed.

For mouse experiments, mice completely deficient in TRα (TRα(0/0)) or TRβ (TRβ(−/−)), generated respectively as described (6, 16) and independently bred, were used together with wild-type mice of the same strain. TRα(0/0) mice lack TRα1, TRα2, TRβ1α, and TRβ2α. TRβ(−/−) mice lack TRβ1 and TRβ2. Hypothyroidism was induced by administration of 0.02% 2-mercapto-1-methylimidazole and 1% sodium perchlorate in the drinking water to adult male mice for 8 wk. Hormonal treatment was accomplished by daily intraperitoneal injections of T3 (2 μg/100 g body wt) for 4 days, as described (16), and experiments were terminated 4–5
h after the last injection. The control groups of animals received only vehicle injections.

**Protein extraction and Western blot analysis.** Total liver extracts were obtained either by homogenizing in medium [210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 5 mM Tris-HCl, pH 7.5, plus protease inhibitors (PMSF, E-64, peptatin A)] or in boiling medium (65 mM Tris·HCl, pH 6.8, 10% glycerol, 0.14 M mercaptoethanol, 2% SDS), followed by centrifuging at 17,400 g for 20 min (22). Proteins (25–60 mg) were separated in 10% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA). The Western blots were probed with monoclonal α- (Sigma, T9026; St Louis, MO) and β-tubulin (isotypes I and II, Sigma T8535; NeoMarkers MS 582-P; Fremont, CA) and polyclonal actin (Sigma, A2026) antibodies. Protein disulfide-isomerase polyclonal antibody (19) was used to control load. As secondary antibodies, anti-mouse and anti-rabbit peroxidase conjugates (Bio-Rad, Hercules, CA) were employed. The immunoreactive proteins were visualized by chemiluminescence (ECL detection system; Amersham, Buckinghamshire, UK).

**RNA extraction and Northern analysis.** Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) from frozen mouse liver and brain samples. Twenty micrograms of RNA were separated on 1.5% (wt/vol) agarose-formaldehyde gels and transferred onto Zeta-Probe blotting membranes (Bio-Rad) with 10× SSC (1× SSC = 150 mM NaCl and 15 mM sodium citrate, pH 7.0). Hybridization was accomplished in 7% SDS and 0.25 M sodium phosphate, pH 7.4 or alternately, when β-tubulin probes were used, in UltraHyb hybridization buffer (Ambion, Austin, TX) that effectively increases hybridization signal. Probes for β-tubulin isotypes 2 (II) and 5 (I) (21), β'-deiodinase type 1 (3), and 7S (1) were labeled with [32P] by use of a random priming system (Amersham).

**Histological methods and immunofluorescence studies.** Mice under anesthesia were perfused either transcardially or by cannulation of the anesthetic gases. Liver specimens were prepared for histological examination, dried, and stored at 4°C. The sections were permeabilized by a vibratome directly from the fixed samples. Microtubular structure was analyzed by immunofluorescence and confocal microscopy. Sections of liver tissue were fixed with a 4% paraformaldehyde in PBS solution, pH 7.4, and also with a 10× SSC buffer solution, 150 mM NaCl and 15 mM sodium citrate, pH 7.0. Hybridization was accomplished in 7% SDS and 0.25 M sodium phosphate, pH 7.4 or alternately, when β-tubulin probes were used, in UltraHyb hybridization buffer (Ambion, Austin, TX) that effectively increases hybridization signal. Probes for β-tubulin isotypes 2 (II) and 5 (I) (21), β'-deiodinase type 1 (3), and 7S (1) were labeled with [32P] by use of a random priming system (Amersham).

**RESULTS**

**Tubulin expression is tissue specific in mammals.** Although tubulin expression is generally considered constitutive and either α- or β-tubulin subunits are frequently used as load controls, their expression is not homogeneous among mammalian tissues. Tubulin expression is highly tissue specific in the mouse, with α- and β-proteins being most abundant in brain and testis (Fig. 1). The same relative results were obtained from rat tissues (not shown).

**Expression patterns of tubulin subunits do not vary in parallel during liver postnatal development: regulation by TH.** The analysis of tubulin levels during postnatal development in the rat liver indicated that the two subunits’ ratio changes in the postnatal days. The β-tubulin subunit level increased steadily from the first day after birth to obtain the adult value (Fig. 2). On the contrary, the α-subunit was abundant after birth, decreasing afterward to reach a level similar to that of the adult value from day 14 onward. The results indicate that the steady-state expression of the two tubulin subunits can be unbalanced in physiological conditions.

We also analyzed the expression patterns of both subunits in rat neonates born from hypothyroid mothers (Fig. 2). The patterns of α- and β-subunits were similar during the postnatal period but different from the corresponding one observed with the normal animals. Protein disulfide-isomerase, a protein whose expression is not affected by thyroid status, was determined in order to allow for load control. These results indicated that TH regulates tubulin expression in the postnatal development of rat liver.

**TH administration increases tubulin expression in adult rat liver.** We also investigated whether tubulin expression in the adult rat liver was sensitive to TH administration. The analysis of tubulin subunits expression in the liver of normal, hypothyroid and T3-treated hypothyroid rats indicated that T3 increased significantly the β-subunit protein level (Fig. 3A). Protein disulfide-isomerase determination was used to control protein load. The T3 status of the three groups of animals was confirmed by determining the mRNA levels of 5'DI (deiodinase), a T3-responsive gene (Fig. 3B). The results thus indicate that

![Fig. 1. Tubulin protein expression in adult mouse tissues. Total tissue extracts were obtained, and Western analysis was performed as described in experimental procedures. β- and α-Tubulin proteins in different mouse adult tissues are expressed at relative similar levels. Protein disulfide-isomerase (PDI) is not included, as in other Western blot figures, to control protein load, because its expression is tissue specific. L, K, B, H, M, Lu, and T stand, respectively, for liver, kidney, brain, heart, skeletal muscle, lung, and testis. Very similar Western blots were obtained with rat tissues, although the signal was lower (not shown). Two independent experiments, for each mouse and rat tissue, were carried out with the same results.](http://ajpendo.physiology.org/doi/abs/10.1152/ajpendo.00414.2004)
β-tubulin expression in the adult rat liver is sensitive to TH administration.

Tubulin expression in wild-type mice and mice lacking TRα or TRβ: effect of TH deprivation and T3 administration. To investigate the role of TRs in the expression of tubulin, we measured the amounts of both subunits in wild-type mice and in mice of the same strain lacking TRα or TRβ (Fig. 4). The results indicated that β-tubulin protein, compared with the wild type, appears to be significantly lower in mice with TRβ deletion. The three types of mice were made hypothyroid and the levels of tubulin subunits determined. TH deprivation resulted in significant decrease of β-tubulin expression in wild-type and in TRα-lacking mice, but surprisingly, a significant increase existed in the mice without TRβ. The effects observed appeared to be specific for β-tubulin subunit, as little effect was seen with α-tubulin subunit or with actin, another cytoskeletal protein. Protein load was controlled with protein disulfide-isomerase. T3 injected into hypothyroid mice lacking TRβ (Fig. 5) decreased the β-tubulin level down to about that of normal mice. Therefore, the results indicate that the effects of TH deprivation on the liver β-tubulin expression can be reversed by T3 administration.

The results suggest that liver β-tubulin expression in vivo is primarily under TRβ control. However, the fact that hypothyroidism and T3 treatment increased or decreased, respectively, β-tubulin levels in TRβ−/− animals suggests that β-tubulin might also be upregulated by unliganded TRα.

TH regulates β-tubulin expression at the mRNA level. To investigate whether TH regulates β-tubulin expression at the mRNA level, RNA was isolated from the livers of wild-type mice and normal, hypothyroid, and hypothyroid T3-treated wild-type animals and animals lacking either TRα or TRβ.
Northern blot containing RNA from the livers of normal and hypothyroid wild-type mice, TR\textsuperscript{+/+} or TR\textsuperscript{−/−} deleted mice (Fig. 6A) was hybridized with the labeled cDNA probe of 5\textsuperscript{−}-deiodinase, a control TH-responsive gene. The abundance of the specific mRNA observed in each sample reproduced previous results from the laboratory where the knockout animals were generated (16). The signal of the TR\textsuperscript{+/+}-deleted mice was higher than that of the wild type and the signal of the TR\textsuperscript{−/−}-deleted mice much lower than the other two, whereas the hypothyroid animals of any type gave no detectable signal. This allowed confirming the status, both genetic and hormonal, of the animals used. The 5\textsuperscript{−}-deiodinase probe used was the liver-specific isotype 1, and for that reason, no signal was observed in the lane containing RNA from wild-type brain. Brain RNA was included in the Northern blots as a sensitivity test of the assay because the liver β-tubulin message appeared to be rare and could be detected only with the aid of a commercial enhancer of hybridization (see EXPERIMENTAL PROCEDURES).

Identical results were obtained with probes for mRNA isotypes 2 or 5, although the signal with the isotype 5 probe was always lower. The results indicating that the increase in β-tubulin protein expression produced by TH deprivation in TR\textsuperscript{−/−}-deleted mice can be reversed by T\textsubscript{3} administration (Fig. 5) were also reproduced at the mRNA level. As shown in Fig. 6B, the relatively high level of β-tubulin message observed in the TR\textsuperscript{−/−}-deleted hypothyroid mice was significantly decreased to mRNA in the liver comprises two mRNA isotypes (2 and 5, or II and I) which are also present in the brain (13). In Fig. 6A, brain RNA showed a strong signal, whereas in liver RNA the signal was clearly detected only in the liver of the hypothyroid TR\textsuperscript{−/−}-deleted mice. These results roughly parallel those obtained with the Western blots (Fig. 4), in that the strongest signal was observed with the hypothyroid TR\textsuperscript{−/−}-deleted mice. However, the modulation in the protein signal of the other samples seen in Western blots was not detected at the mRNA level, probably because the liver β-tubulin message was too scarce except in the case of the hypothyroid TR\textsuperscript{−/−}-deleted mice. Identical results were obtained with probes for mRNA isotypes 2 or 5, although the signal with the isotype 5 probe was always lower. The results indicating that the increase in β-tubulin protein expression produced by TH deprivation in TR\textsuperscript{−/−}-deleted mice can be reversed by T\textsubscript{3} administration (Fig. 5) were also reproduced at the mRNA level. As shown in Fig. 6B, the relatively high level of β-tubulin message observed in the TR\textsuperscript{−/−}-deleted hypothyroid mice was significantly decreased to...
about the same levels of the euthyroid animals upon T₃ treatment.

Our mRNA results indicate that, at least in the TRβ-deleted mice, TH regulates expression of liver β-tubulin at the mRNA level.

Analysis of liver structure by section staining and of microtubular structure by immunofluorescence confocal microscopy, in wild-type mice and TRα- or TRβ-deleted mice. The differences in the amounts of β-tubulin (Fig. 4) found in wild-type mice and in mice lacking TRs, either TRα or TRβ, prompted us to analyze whether the liver structure of these animals was affected. The toluidine blue staining of liver sections (Fig. 7) indicated that, by this criterion, the livers of the three types of animals were apparently normal. We also analyzed the organization of the microtubular cytoskeleton by immunofluorescence confocal microscopy using anti α- and β-tubulin antibodies (Fig. 8). In the wild-type mice, the characteristic microfibrillar arrays of the tubulin cytoskeleton were observed with both antibodies. In agreement with Western blot data (Fig. 4), in mice lacking either TRα or TRβ, the amount of signal with α-tubulin antibody was similar to that observed in the wild-type mice. However, the cytoskeleton appeared somewhat disorganized, punctuated, and with less fibers. The β-tubulin antibody also gave rise to a punctuated pattern in the mice lacking TRα. In the mice without TRβ, the signal obtained with the β-tubulin antibody was in addition very low, in coincidence with the Fig. 4 data. This indicated that the microtubular structure was not only disorganized but clearly decreased in the mice lacking TRβ. The results suggest that both TRs are required for the microtubular organization observed in the wild-type liver and that β-tubulin expression, as also indicated from Western experiments data (Fig. 4), is critically controlled by TRβ.

DISCUSSION

Tubulin protein expression in rat and mouse tissues is tissue specific, with both subunits coordinately expressed in the adult tissues (Fig. 1). However, we have observed that the two subunits’ ratio changes in the liver during the postnatal develop-

Fig. 5. Increased protein expression of tubulin β-subunit in the livers of TRβ-deleted hypothyroid mice is reduced upon T₃ administration. Animals were treated as indicated in EXPERIMENTAL PROCEDURES. Total protein extracts obtained from liver of N, H, and H+T₃ mice were subjected to analysis performed as indicated in the same section. Western blots were probed with anti-tubulin β- and α-subunits and actin to check for other cytoskeletal protein and with PDI to check for protein load. Each lane corresponds to a different animal. Three to four animals were analyzed for each group. The significant differences of β-tubulin in the hypothyroid TRβ⁻ mice are marked, **P < 0.01.

Fig. 6. TH regulates liver tubulin β-subunit at the mRNA level. Total RNA was isolated from the brain of wt mice, and livers of wt mice and mice lacking TRα or TRβ in the different N, H, and H+T₃ hormonal states, and Northern analysis was performed as indicated in EXPERIMENTAL PROCEDURES. Brain RNA (B) was included to compare levels of expression in the 2 tissues. Mouse 5′DI-1 probe was used to identify mice genetically and hormonally (16), and 7S to control RNA load. A: results obtained with RNA from wt mice and mice lacking TRα or TRβ, both N and H, are presented. B: results are from mice lacking TRβ, from N, H, and H+T₃ animals; 2 different animals per group are shown. Two independent experiments were carried out with the same results; 4 animals per group were analyzed in total. Statistical analysis indicated that the hypothyroid TRβ⁻ mice were significantly different from both N and H+T₃ TRβ⁻ mice (*P < 0.05).
opment (Fig. 2), with the α-subunit being relatively high with respect to the adult value and the β-subunit low, on the first day after birth. Although it is known that tubulin expression is regulated by TH in the brain (15), regulation in the liver has not been investigated. The analysis of α- and β-subunit protein levels in the livers of hypothyroid rat neonates indicated that T₃ affects the developmental patterns of both subunits during postnatal development. The β-subunit shows a sustained increase from birth to the adult stage, a pattern that is altered in hypothyroidism. Regarding α-tubulin, it is interesting to observe that, from 10 days after birth, changes in α-tubulin expression in hypothyroid newborns are quite similar to those described just after birth for normal animals. This result agrees well with a delayed development of hypothyroid fetuses and newborns, an observation found with other genes regulated during the perinatal period (2). The different patterns of tubulin subunit expression may be related to the developmental regulation of TRs. T₃ binding capacity in the fetal liver is solely accounted for by TRα (22). TRβ starts being expressed after birth and, by 15 days of age, it represents one-third of the total T₃ capacity, which increases to 80% in the adult liver (20). We also investigated whether tubulin expression in the adult rat liver was sensitive to TH administration by analyzing tubulin subunits expression in normal, hypothyroid, and T₃-treated hypothyroid rats. The results indicated that TH mainly activated β-tubulin expression. The observation that the hypothyroid rats showed normal levels of β-tubulin and that the T₃ effect was only evident after T₃ supplementation of these animals resembles the behavior of other T₃-responsive genes that respond only to high T₃ doses (5). The reductions in protein and mRNA levels of β-tubulin 2 and 5 isoforms were reported in the brain of congenitally hypothyroid hyt/hyt mice (4). β-Tubulin 2 and 5 isotypes are also present in the liver. To analyze the role of both TRs in tubulin expression, we measured tubulin levels in mice lacking either all the TRα isoforms (16) or TRβ1 and TRβ2 (6). The relative contributions of TRα1 and TRβ in the liver of mice are similar to those in the rat (15, 16). The results suggest that T₃ regulation of β-tubulin is mediated mainly through TRβ. However, the fact that hypothyroidism and T₃ treatment increased or decreased, respectively, β-tubulin levels in TRβ−/− animals suggests that β-tubulin might also be upregulated by unliganded TRα. The results on tubulin expression during rat liver postnatal development can be better interpreted in light of the results obtained with the knockout mice. The weak signal observed with β-tubulin in the neonates and the pattern of expression afterward (Fig. 2) paralleled the expression of TRβ (20). α-Tubulin is probably under TRα control in the fetus, as suggested by the repressed expression in hypothyroid neonates (Fig. 2) and by the fact that fetal liver contains only TRα1 (15). Our results support previous data in mutant mice (16) indicating that deficiency in one of the TR genes is not compensated for by the other. The mRNA analysis of these mice indicated that TH regulates β-tubulin at this level. The relatively high mRNA level observed with hypothyroid TRβ-deprived mice was reduced to the euthyroid level after T₃ treatment (Fig. 6). However, the undetectable levels of the β-tubulin messages in the other animal types precluded the comparative analysis with the wild-type and TRα-deleted mice.

Although the histological examination of liver structure in wild-type and mutant mice indicated no obvious differences, the microtubular organization showed alterations. The immunofluorescence analysis of liver sections from animals of each type using anti-tubulin α- and β-subunit antibodies (Fig. 8) indicated that, compared with the wild type, and in agreement with the results of Western analysis (Fig. 4), the signal given by the anti-β-subunit was very low in the animal without TRβ. In the same animal, the α-subunit signal was high, as observed in Western blots (Fig. 4), but appeared punctuated, indicating low polymerization, likely due to the lack of the β-subunit required to form the heterodimers. Similar images were observed with β-tubulin mutants expressed in tissue culture cells.
where they failed to polymerize with endogenous α-tubulin (26). Unexpectedly, despite the high expression of both subunits in the liver of TRα-depleted mice (Fig. 4), the microtubular network showed decreased polymerization with both subunit antibodies, suggesting that TRα may be involved at a certain level in achieving polymerization or in modulating microtubule dynamics. Multiple factors seem to be involved in the synthesis, transport, and storage of α- and β-tubulin in the folding of the heterodimers and the polymerization, stabilization, or dynamics of microtubules (14). In the TRβ-deleted mice, the distinctive punctuated α-tubulin immunofluorescence pattern may indicate that folding is affected. A similar punctuated pattern was described for free α-hemoglobin precipitated when expressed in cell experiments in the absence of the specific chaperone (11). The accumulation of unfolded protein and protein aggregates is detrimental and may lead to cell death and, to limit this accumulation, the cell activates a specific stress-signaling pathway (10).

To summarize, our results indicate that TH regulates tubulin expression in the mammalian liver. In studies with knockout mice, specifically, β-tubulin appears to be critically controlled by TRβ, with unliganded TRα probably also upregulating expression. It is suggested that both TRs may be required for the microtubular organization observed in the wild-type liver.

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