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Difference in the Susceptibility of Two Phenobarbital-Inducible Forms, P450IIB1 and P450IIB2, to Thyroid Hormone- and Growth Hormone-Induced Suppression in Rat Liver: Phenobarbital-Inducible P450IIB2 Suppression by Thyroid Hormone Acting Directly, But Not Through the Pituitary System

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SUMMARY

Suppression of two major phenobarbital-inducible cytochrome P-450s, P450IIB1 and P450IIB2, by thyroid hormone was studied and compared with growth hormone (GH)-induced suppression in rats *in vivo* and hepatocytes in primary culture *in vitro*. Treatment of adult male rats with 50 μg/kg triiodothyronine (T_3) reduced the constitutively expressed amounts of P450IIB1 (up to 1 pmol/mg of protein) and P450IIB2 (2–5 pmol/mg of protein) to 42% and 3% of their levels in nontreated controls. Thyroidectomy increased the hepatic contents of P450IIB2 (to levels of 50–80 pmol/mg of protein) and, to a lesser extent, P450IIB1 (1–5 pmol/mg of protein) in male and female rats. Supplement of T_3 to thyroidectomized rats reversed the increased contents to levels similar to those observed in normal rats. Hypophysectomy also increased both P450IIB1 and P450IIB2 protein, and their levels in both sexes were similar to that of P450IIB2 in thyroidectomized rats. Treatment of hypophysectomized rats with T_3 as well as human GH suppressed hepatic contents of P450IIB1 and P450IIB2. In a hepatocyte culture including 2 mM phenobarbital, T_3 and GH suppressed both P450IIB1 and P450IIB2. Other thyroid hormone derivatives, including thyroxine, D-T_3, and reversed T_3, also showed suppressive effects, in parallel with the potencies for their stimulatory action that have been reported. These results indicate that thyroid hormone may suppress both P450IIB1 and P450IIB2 by a direct effect on the liver, but not by an indirect effect through the modulation of pituitary GH synthesis. The high susceptibility of hepatic P450IIB2 to thyroid hormone-induced suppression also indicates that constitutive and phenobarbital-induced levels of P450IIB2 are suppressively regulated preferentially by thyroid hormone, in contrast to the high susceptibility to GH of P450IIB1 in rat liver. In addition, a difference in the suppressive mechanisms of thyroid hormone and GH was suggested by the difference in susceptibility to cycloheximide.

P-450,[1] which catalyzes the oxidation of structurally diverse chemicals, constitutes a large number of hemoproteins (2, 3). Recent studies on molecular cloning of P-450s have made it possible to categorize the genes into families and subfamilies on the basis of the primary structures (1). P450IIB1 and P450IIB2, which are also termed, respectively, P-450b and P-450e, are known as major phenobarbital-inducible forms in rat liver (4, 5). These forms share a high degree of similarity, not only in their primary structures (6, 7) but also in their genetic and catalytic properties and responses to chemical inducers (8–11).

In the regulation of the P450IIB subfamily, phenobarbital has been shown to induce these P-450s by stimulation of gene transcription (12, 13). Differences in tissue specificities for expression have also been reported (14–17). However, no clear mechanism has been presented that shows how phenobarbital and other chemicals activate the gene transcription. In our previous papers (18, 19), pituitary GH was shown to suppress both constitutive and phenobarbital-induced expression of hepatic P450IIB1 and P450IIB2 in rats.

Secretion of GH in rats is different between the sexes; in the male rat GH is secreted in regular episodes, with low levels between peaks, whereas in the female GH is secreted frequently, producing irregular peaks and higher basal levels (20). Although microsomal contents of P450IIB1 and P450IIB2 are low in normal adult rats, their levels are increased up to ~100-fold in GH-depleted states (18, 21). In addition, increased levels of

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[1]For individual P-450 forms, a recently described nomenclature based on gene family was used (1).

ABBREVIATIONS: P-450, cytochrome P-450; T_3, triiodothyronine; GH, growth hormone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; T_4, thyroxine; T_3-Ac, triiodothyroacetic acid; r-T_3, reversed T_3.

P450IIB1 and P450IIB2 in hypophysectomized rats were reduced by either intermittent injection or continuous infusion of human GH (which mimicked the male and female secretory patterns, respectively), although more of a decrease was observed with the continuous infusion. Consistent with the sex-related difference in GH secretory profile, the hepatic content of constitutively expressed P450IIB1 is higher in male than female adult rats. However, no clear sex-related difference was detected in the level of P450IIB2, suggesting the role of an unidentified factor in the regulation of P450IIB forms. We have recently shown that thyroid hormone suppresses P450IIB1 and P450IIB2 in hypophysectomized rats (22). Thyroid hormone is known to activate the GH gene at the pituitary (23). These results suggest the involvement of thyroid hormone in the expression of P450IIB1 and P450IIB2. Therefore, the role of thyroid hormone and its interaction with GH in the activation of P450IIBs were investigated *in vivo* and *in vitro*, using hepatocytes in primary culture. The data obtained indicate a clear difference in susceptibility of P450IIB1 and P450IIB2 to thyroid hormone-induced suppression, although both forms are under the influence of thyroid hormone as well as GH in rat liver.

Materials and Methods

Chemicals. Alkaline phosphatase-conjugated anti-rabbit IgG (whole molecule), nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, T_3, D-T_3, thyroxine, reversed T_3, triiodothyroacetic acid, cycloheximide, somatotropin (GH), and *n*-propylthiouracil were obtained from Sigma Chemical Co. (St. Louis, MO). Collagenase was obtained from Wako Pure Chemical (Osaka). Human GH used for *in vivo* experiments was a generous gift from Sumitomo Pharmaceutical (Osaka, Japan).

Treatment of animals. Sprague-Dawley rats were obtained from Clea Japan (Tokyo). Animals were thyroidectomized at 7 weeks of age, under pentobarbital anesthesia, and left to recover for 1 week (24). To prevent loss of calcium, calcium lactate was included at 1% concentration in the drinking water. Some of the animals were treated with a 5 or 50 μg/kg dose of T_3 intraperitoneally once a day for 7 days. *n*-Propylthiouracil was given dissolved in drinking water (0.1 mg/ml) for 14 days to 7-week-old male rats. These animals were separated into six groups: 1) no treatment, 2) treated subcutaneously with 5 μg/kg T_3 once a day for 7 days, 3) treated subcutaneously with 50 μg/kg T_3 once a day for 7 days, 4) treated subcutaneously with 50 μg/kg T_3 once a day for 3 days, 5) treated intraperitoneally with 50 mg/kg phenobarbital once a day for 3 days, and 6) treated subcutaneously with 50 μg/kg T_3 and intraperitoneally with 50 mg/kg phenobarbital once a day for 3 days.

Hypophysectomy was performed at 7 weeks of age, and the animals were given a subcutaneous injection (2 IU/kg) twice a day or osmotic infusion (0.01 IU/hr) of GH for 7 days, as reported previously (25). Some hypophysectomized rats were given T_3 subcutaneously, at 5 or 50 μg/kg, for 7 days.

Primary hepatocyte culture. Matrigel was prepared from Engelbreth-Holm-Swarm sarcoma propagated in C57BL/6J female mice, following the method of Schuetz *et al.* (26), and was stored at −20°. Matrigel kept at 4° overnight was applied in 500 μl to plastic 10-cm dishes. Gelation was completed within 30–60 min at room temperature. For hepatocyte preparation, adult male Sprague-Dawley rats (body weight, 200–250 g) were used. The livers were perfused *in situ* for 10–15 min with 0.5 mM EGTA solution, through the portal vein of ether-anesthetized rats, followed by a Waymouth 752/1 modified medium containing amino acids, salts, vitamins, minerals (zinc, and selenium), and insulin (1.5×10^{-7} M) as the only hormone (26), plus 0.028% collagenase, without recirculation of the perfusate with collagenase. After 12–15 min, the liver was carefully cut from its ligaments, transferred to a sterile bottle, opened with scissors, and shaken with fresh collagenase solution in a water bath at 35°. After filtration through a double layer of sterile cotton gauze, the suspension was centrifuged at $60 \times g$ for 2 min. Cloudy supernatant over the well defined cells was aspirated and discarded. Two cycles of washing with basal medium (without serum or hormones) were carried out, and the cells were suspended finally in complete culture medium. Approximately 1×10^7 cells in a final volume of 8 ml were plated in a 10-cm culture dish. The cells were distributed evenly and allowed to settle to form a single contiguous layer on the surface of the plate. Cultures were maintained in a humidified incubator at 35°, in an atmosphere of 5% CO_2/95% air, and adjusted to bring the pH of the medium to 7.30–7.40. At 24-hr intervals thereafter, the culture medium was renewed, with some inducers added as described in the experimental protocols.

Immunochemical quantification. Microsomes were prepared as previously described (27). For *in vitro* experiments, hepatocytes were washed with 100 mM potassium phosphate buffer (pH 7.40), harvested by scraping with a cell lifter (Costar), pelleted, and then suspended in 1 ml of 100 mM potassium phosphate buffer (pH 7.40), 120 or 168 hr after culture. Thereafter, microsomal samples were solubilized with a buffer containing 13.5% 2-mercaptoethanol and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, according to the method of Laemmli (28), using 7.5% gels for analysis. Microsomal proteins were electrophoretically transferred to nitrocellulose paper (Bio-Rad) and probed with rabbit antibodies raised against P450IIB1, which recognize both P450IIB1 and P450IIB2. Characteristics of purified P-450 preparations and their antibodies were reported previously (29–31). P450IIB1 and P450IIB2 correspond to P-450b and P-450e, respectively (4, 32), and these P-450s belong to the P450IIB subfamily (1). For the quantitation of P450IIIA, antibodies raised against P-$450_{6s\text{-}1}$ (33, 34) were used. Alkaline phosphatase activity on immunoblot was quantitated essentially by the method of Blake *et al.* (35).

RNA blots. Total RNA was prepared by the guanidine thiocyanate method of Chirgwin *et al.* (36). Hepatocytes were harvested by scraping with a cell lifter in 1 ml of 4 M guanidine thiocyanate solution per dish. Hepatocytes from two dishes (2×10^7 cells) were pooled, and total RNA was prepared as previously described (36). The amount of total RNA was determined from the absorbance at 260 nm, using a Beckman DU-65 spectrophotometer. For slot blotting, 10 or 20 μg of total RNA were blotted according to the procedure recommended by the supplier (Minifold II; Schleicher & Scull Inc.) (37). For Northern blotting, 10 or 20 μg of total RNA were fractionated in a 1.2% formaldehyde-agarose gel (38) and transferred to nylon paper (Gene Screen; NEN). The filter was hybridized with ^{32}P-labeled oligonucleotide or cDNA for P450IIB1 or β-actin, as reported previously (37). The oligonucleotide has a sequence of 3′-d(AGTGTGGCCGATGGTTGG)-5′, which was used by Giachelli and Omiecinski (39) to detect selectively P450IIB1 mRNA. A *Bam*HI fragment of P450IIB2 cDNA (525-base length) was also used for the estimation of P450IIB1/IIB2 mRNA. After exposure to X-ray film, the radioactive spot was counted using a liquid scintillation counter. Quantification was carried out using the level of β-actin mRNA as the internal standard.

Other assay methods. Protein concentration was determined by the method of Lowry *et al.* (40), with bovine serum albumin as the standard. Malic enzyme activity of hepatocyte cytosols was determined by the modified method of Hsu and Lardy (41). The reaction mixture consisted of 0.025 M sodium-potassium phosphate buffer (pH 7.40), 4 mM magnesium chloride, 2.23 mM $NADP^+$, 0.5 mM *l*-malic acid, and 0.1 mg of cytosol, in a final volume of 500 μl. The reaction was started by the addition of $NADP^+$ and monitored as the change in absorbance at 340 nm, using a Beckman DU-65 spectrophotometer. The enzyme activity was expressed as units per mg of protein (1 unit = reduction of 1 nmol of $NADP^+$/min). The reaction was linear for 5–6 min with the experimental cytosol concentrations. Statistical significance was determined using Student's *t* test.

Results

Effect of T_3 treatment on hepatic P450IIB1 and P450IIB2 contents in normal male rats. As reported previously (18), P450IIB1 was detectable in low amounts in livers of normal adult male Sprague-Dawley rats (Table 1). The level was decreased by treatment with T_3 (50 μg/kg for 7 days) to 42% of the normal control. The decrease was more profound in the P450IIB2 content; only 3.2% of the nontreated control level was detected in T_3-treated male rats. Thus, the ratio of microsomal P450IIB2 to P450IIB1 contents in the liver was changed from 4.9 in control to 0.4 after T_3 treatment. In female rats, P450IIB1 was not detectable in either control or T_3-treated animals. P450IIB2 was detectable in control female rats but was suppressed by T_3 to an undetectable level.

P450IIB1 and P450IIB2 under hypothyroid conditions. Propylthiouracil is known to decrease plasma thyroid hormone levels by inhibiting the synthesis of thyroid hormone at the thyroid gland (24). As shown in Table 2, hepatic contents of P450IIB1 and P450IIB2 were increased 7-fold and 15-fold, respectively, in adult male rats treated with propylthiouracil for 2 weeks. Similar increases of P450IIB1 (5-fold) and P450IIB2 (16-fold) were also observed after thyroidectomy of male rats. Treatment of female rats with propylthiouracil or thyroidectomy increased the content of P450IIB1 from undetectable to a level similar to that observed in untreated male rats. Thus, the content of P450IIB1 was still 2–10-fold higher in male than in female hypothyroid animals. However, the hepatic content of P450IIB2 was elevated more than 20-fold in both propylthiouracil-treated and thyroidectomized female rats, and the sex-related difference disappeared in hypothyroid conditions. Treatment of thyroidectomized female rats with 5 μg/kg T_3 suppressed P450IIB1 and P450IIB2 to an undetectable level and 28% of the level in thyroidectomized female rats, respectively. Further, stronger suppression was detected after treatment with 50 μg/kg T_3; microsomal contents of P450IIB1 and P450IIB2 in thyroidectomized male and female rats were decreased to levels comparable to or less than the contents in normal rats.

The effect of thyroid hormone on phenobarbital induction of P450IIB1 and P450IIB2 was also examined with propylthiouracil-treated male rats. Microsomal contents of P450IIB1 and P450IIB2 were increased by treatment of normal and propylthiouracil-treated rats with phenobarbital (50 mg/kg for 3 days) (Fig. 1). Pretreatment with propylthiouracil caused a slightly higher induction of P450IIB2 in phenobarbital-treated rats ($p < 0.05$), although the level of P450IIB1 was not significantly different with or without the advanced propylthiouracil treatment. Furthermore, the co-administration of T_3 to propylthiouracil-treated rats diminished the phenobarbital-induced amount of P450IIB2 ($p < 0.05$), but only slightly reduced the amount of P450IIB1 ($p < 0.05$).

Contents of P450IIB1 and P450IIB2 under GH-depleted conditions. As shown in Table 3, hepatic contents of P450IIB1 and P450IIB2 were increased 58-fold and 16-fold, respectively, after hypophysectomy of male adult rats. Similar increases were also observed after hypophysectomy of female rats. Therefore, microsomal contents of P450IIB1 and P450IIB2 were 15–60-fold higher in hypophysectomized than in normal adult rats, and the absolute levels of both forms were similar in male and female hypophysectomized rats. Microsomal contents of P450IIB2 in both sexes of thyroidectomized rats were comparable to those of P450IIB2 in hypophysectomized rats, although the content of P450IIB1 was much lower in thyroidectomized than in hypophysectomized rats (Table 2

TABLE 1

Microsomal P450IIB1 and P450IIB2 contents in normal and T_3-treated rats

Microsomal contents of P450IIB1 and P450IIB2 were quantitated by Western blots. Data are presented as the mean ± standard deviation of the number of individual animals indicated in parentheses. Rats were treated with 50 μg/kg T_3 once a day for 7 days.

Treatment	P450IIB1	P450IIB2
	pmol/mg of protein	
Male		
Control (3)	0.66 ± 0.14 (100)[a]	3.20 ± 0.69 (100)
Plus T_3 (3)	0.28 ± 0.02[b] (42.4)	0.11 ± 0.04[b] (3.4)
Female		
Control (3)	ND[c]	2.34 ± 0.49
Plus T_3 (4)	ND	ND

[a] Values relative to male controls, set as 100.
[b] Significantly different from the respective control ($p < 0.05$).
[c] ND, <0.05 pmol/mg of protein.

TABLE 2

Effect of propylthiouracil treatment and thyroidectomy on microsomal contents of P450IIB1 and P450IIB2

Experimental details are described in Materials and Methods. Data are expressed as mean ± standard deviation. Numbers of the animals used are shown in parentheses.

Treatment	P450IIB1	P450IIB2
	pmol/mg of protein	
Male		
Control (4)	0.82 ± 0.27	4.87 ± 0.84
PTU[a] (5)	5.55 ± 0.58	70.79 ± 7.66
Thyroidectomized (4)	4.02 ± 0.74	77.02 ± 10.59
Thyroidectomized plus T_3 (50 μg/kg) (4)	0.90 ± 0.14	2.48 ± 0.28
Female		
Control (3)	ND[b]	2.34 ± 0.49
PTU (4)	0.42 ± 0.17	56.11 ± 11.87
Thyroidectomized (4)	1.83 ± 0.53	53.25 ± 11.73
Thyroidectomized plus T_3 (5 μg/kg) (3)	ND	14.39 ± 2.73
Thyroidectomized plus T_3 (50 μg/kg) (4)	ND	ND

[a] PTU, propylthiouracil.
[b] ND, <0.05 pmol/mg of protein.

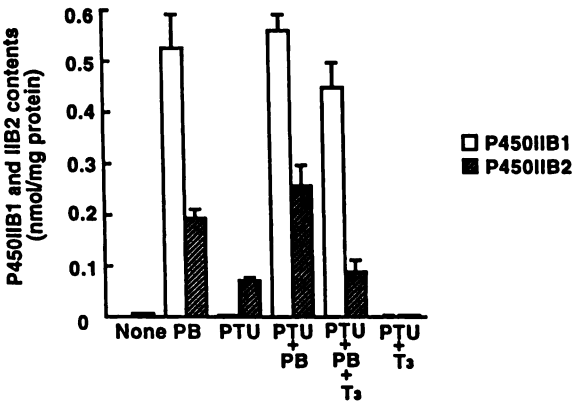

Fig. 1. Effect of propylthiouracil pretreatment on microsomal contents of P450IIB1 and P450IIB2 in rat livers. Data represent the mean ± standard deviation of four or five different male rats. Experimental details for treatments with propylthiouracil (*PTU*), phenobarbital (*PB*), and T_3 are described in Materials and Methods. *None*, nontreated.

TABLE 3

Microsomal contents of P450IIB1 and P450IIB2 in hypophysectomized and GH-treated male and female rats

The numbers in parentheses represent the number of animals used. Data are expressed as mean ± standard deviation. GH(s), human GH given subcutaneously intermittently twice a day for 7 days. GH(i), human GH given by continuous infusion for 7 days.

Treatment	P450IIB1	P450IIB2
	pmol/mg of protein	
Male		
Control (4)	0.95 ± 0.39	3.60 ± 1.58
Hypophysectomized (4)	55.00 ± 7.57	56.60 ± 14.18
plus GH(s) (4)	26.73 ± 2.54	37.80 ± 4.15
plus GH(i) (3)	3.22 ± 1.67	15.13 ± 3.90
Female		
Control (4)	ND[a]	2.76 ± 0.55
Hypophysectomized (4)	51.87 ± 5.97	63.48 ± 9.05
plus GH(s) (3)	34.13 ± 8.10	49.27 ± 8.29
plus GH(i) (3)	7.70 ± 1.10	43.21 ± 6.50

[a] ND, <0.05 pmol/mg of protein.

TABLE 4

Microsomal contents of P450IIB1 and P450IIB2 in T_3-treated hypophysectomized male rats

T_3 was given subcutaneously for 7 days to male hypophysectomized rats. Microsomal contents of P450IIB1 and P450IIB2 were quantitated by Western blots, as described in Materials and Methods.

Treatment	P450IIB1	P450IIB2
	pmol/mg of protein	
None (4)[a]	93.88 ± 10.51 (100)[b]	58.69 ± 2.40 (100)
T_3		
5 μg/kg (3)	77.22 ± 5.15 (82.2)	42.73 ± 4.50 (72.8)
50 μg/kg (4)	3.81 ± 0.23 (4.06)	0.39 ± 0.26 (0.67)

[a] Number of individuals examined.
[b] Values relative to non-treated hypophysectomized male rats, set as 100.

and 3). Supplement of human GH by intermittent injection or continuous infusion, which mimicked, respectively, the secretory pattern of GH in male and female adult rats, reduced hepatic levels of both forms in hypophysectomized rats. However, the GH-induced suppression was less for P450IIB2 than for P450IIB1; hepatic P450IIB2 contents in male and female hypophysectomized rats were only reduced, respectively, to 27% and 68% of hypophysectomized controls by the continuous infusion, whereas the P450IIB1 contents were decreased, respectively, to 6% and 15% of hypophysectomized controls by this treatment.

Thyroid hormone is known to stimulate the synthesis of pituitary GH by the activation of gene transcription (42, 43). However, a thyroid hormone receptor exists in the liver, and T_3 has been shown to evoke the stimulatory signal for liver β-hydroxy-β-methylgultaryl coenzyme A synthase in hypophysectomized rats (44, 45). Therefore, thyroid hormone-induced suppression of P450IIB could be mediated either directly in the liver or indirectly through the pituitary, in which thyroid hormone modulates GH secretion. To distinguish which pathway is responsible for the suppression of P450IIB proteins, the effect of T_3 on P450IIB1 and P450IIB2 was examined, using hypophysectomized male rats. As shown in Table 4, the increased levels of P450IIB1 and P450IIB2 were reversed slightly by the administration of 5 μg/kg T_3 in hypophysectomized rats. In addition, almost complete suppression was observed with administration of 50 μg/kg T_3, suggesting that T_3 may directly affect livers.

Suppression of P450IIB1 and P450IIB2 by thyroid hormone and GH in primary cultured hepatocytes. Effects of T_3 and GH on P450IIB1 and P450IIB2 were investigated with rat hepatocytes in primary culture, using a technique developed recently (26). In this experiment, hepatocytes isolated from adult male rats were plated on a Matrigel-coated dish (10 cm diameter) at the density of 1×10^7 cells/dish. T_3 and/or human GH was added 3 hr after the plating, and phenobarbital was added for the period of 48 hr before harvest. As shown in Fig. 2, the microsomal content of P450IIB1 in rat hepatocytes increased to 110 pmol/mg of protein 48 hr after the addition of 2 mM phenobarbital. Addition of 10 nM T_3 caused the suppression of P450IIB1 protein in approximately one third of the phenobarbital-treated controls. Similar suppression was also detected in 0.05 mU GH-treated cells. In addition, complete suppression was observed in T_3- plus GH-treated cells. P450IIB2 was also induced in this system and was suppressed by T_3 and GH similarly to the case with P450IIB1. The dose dependency of the T_3 effect on P450IIB1 and P450IIB2 was measured as shown in Fig. 3. The suppression was dose dependent in the range of 1 to 100 nM and was nearly complete at 100 nM. Under these conditions, cytosolic malic enzyme was increased with the increase in T_3 concentration (data not shown); this enzyme is known to be positively regulated by thyroid hormone in rat livers (46).

Although T_3 is known to have the highest affinity for the hepatic thyroid hormone receptor, several analogues also bind

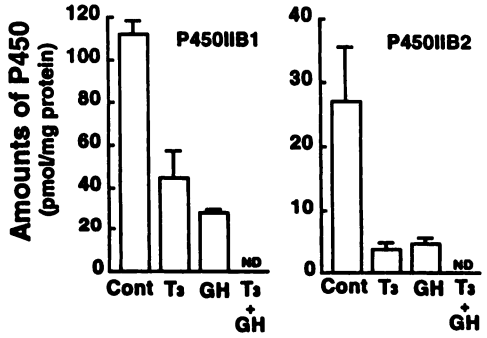

Fig. 2. Suppressive effects of T_3 and GH on the contents of P450IIB1 and P450IIB2 in rat hepatocytes. Phenobarbital (2 mM) was added, 2 days before harvest of cells, to both control (*Cont*) and experimental groups. For the experimental groups, 10 nM T_3 and/or 0.05 mU of GH was also included from 3 hr after the plating until harvest of hepatocytes (120 hr after the plating). *ND*, not detected (<0.05 pmol/mg of protein).

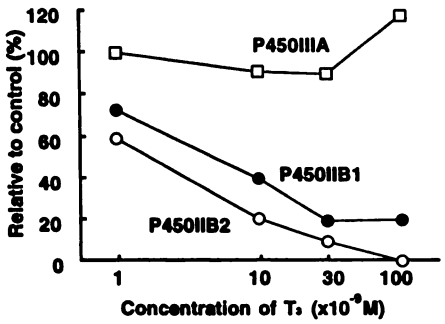

Fig. 3. Dose-dependent suppression of P450IIB1 and P450IIB2 by T_3 in rat hepatocytes. Each *point* represents the mean of values obtained from three different dishes. In this experiment, a IIIA family of P-450 was also quantified, which was also induced by phenobarbital in this system. Other experimental details were the same as described in Materials and Methods and the legend to Fig. 2.

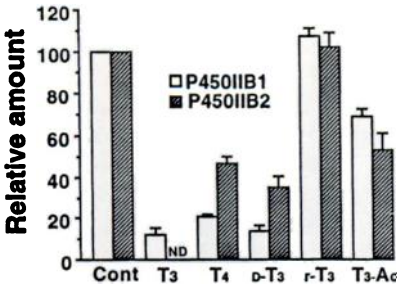

Fig. 4. Effects of thyroid hormone analogues on phenobarbital induction of P450IIB1 and P450IIB2 in rat hepatocytes in primary culture. Experiments were done in triplicate, using 30 nM concentrations of each thyroid hormone derivative in the presence of 2 mM phenobarbital. Other experimental details are the same as described in the legend to Fig. 2.

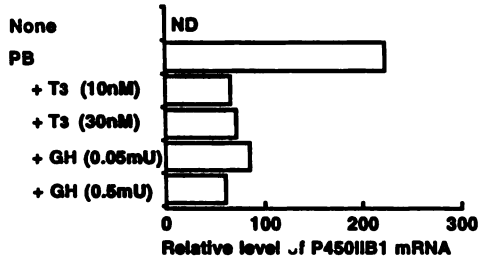

Fig. 5. Effect of T_3 and GH on phenobarbital induction of P450IIB1 mRNA in rat hepatocytes. Total RNA was prepared from two dishes (2×10^7 cells). The data indicate the mean of the values obtained from two or three different samples. Other experimental details are described in Materials and Methods. *PB*, phenobarbital.

the receptor (47). To assess whether the suppression of the P450IIBs is mediated by the thyroid hormone receptor, the effects of T_4, r-T_3, and D-T_3 were compared with that of T_3 (Fig. 4). Consistent with the efficacy of each analogue on the stimulative effect, the microsomal content of P450IIB1 was decreased by the addition of these thyroid hormone analogues. Changes in P450IIB1 mRNA level were also measured, using a specific oligonucleotide probe for P450IIB1 cDNA. As shown in Fig. 5, the P450IIB1 mRNA level in phenobarbital-induced hepatocytes was decreased by the addition of T_3. A similar decrease was also observed with a *Bam*HI fragment of P450IIB2 cDNA (data not shown). Consistent with a recent report using a probe recognizing both P450IIB1/IIB2 cDNAs (48), a clear decrease of the mRNA was also observed with the addition of GH.

In the present study, thyroid hormone and human GH were added 3 days before the addition of phenobarbital to hepatocytes (Fig. 6A). Addition of T_3 or human GH only for the last 48-hr period (Fig. 6B) also showed suppression of microsomal P450IIB1 protein, but to a lesser extent. Although the data are not shown, addition of human GH or T_3 only during the first 72 hr had no clear effect on either P450IIB1 and P450IIB2. Late addition of T_3 or human GH to phenobarbital-pretreated hepatocytes (T_3 or human GH added after 5 days of induction with phenobarbital) did not reduce but rather increased the content of P450IIB1. These results suggest that T_3 and GH did not suppress P450IIB protein by stimulating degradation of the synthesized protein.

To assess the reason why the advance addition of hormone, especially thyroid hormone, is necessary for the maximal suppression of the P450IIBs, an inhibitor of protein synthesis, cycloheximide, was added to hepatocyte cultures intermittently 24 hr before and after addition of phenobarbital, each time for

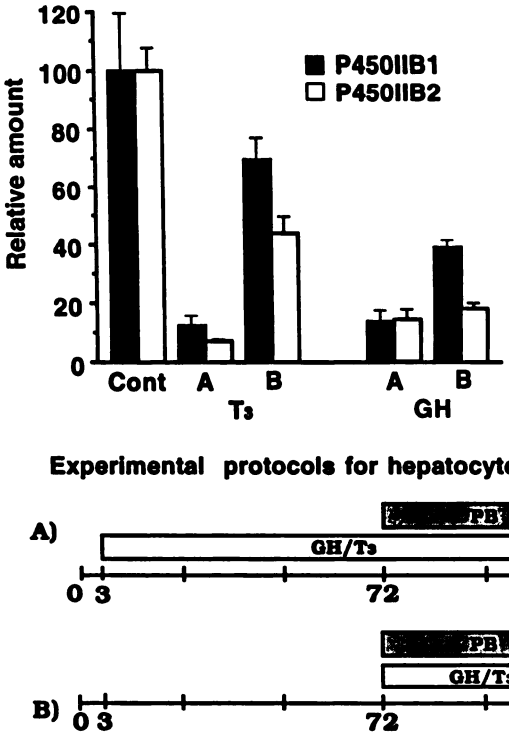

Fig. 6. Effect of changes in the order of addition of T_3 and GH on P450IIB1 and P450IIB2 in rat hepatocytes. In this experiment, 2 mM phenobarbital (*PB*) and 30 nM T_3 or 0.05 mU of GH were used. Relative data, compared with nontreated controls, are shown in this figure. *Column* and *bar*, mean ± standard deviation of the values obtained from three different samples.

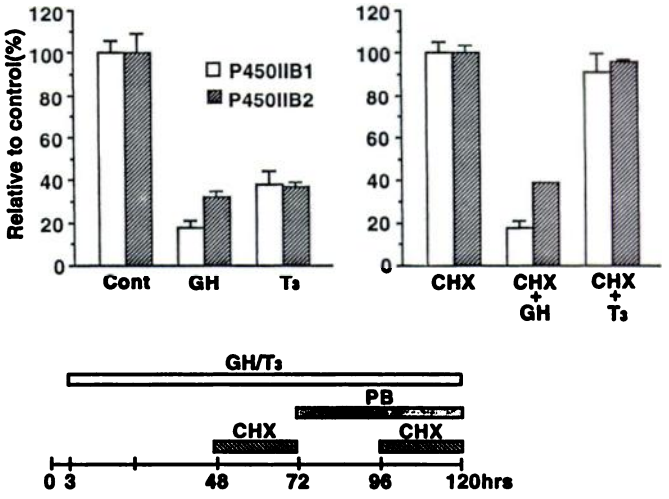

Fig. 7. Effect of cycloheximide on the thyroid hormone- and GH-induced suppression of P450IIB1 and P450IIB2. The experimental protocol for addition of chemicals is shown below the experimental data. In this experiment, 0.35 μM cycloheximide (*CHX*) was used with 0.05 mU of GH or 30 nM T_3. The mean absolute contents of P450IIB1 and P450IIB2 were 61.3 and 24.4 pmol/mg of protein, respectively, in phenobarbital-treated controls and 12.0 and 3.6 pmol/mg of protein, respectively, in phenobarbital- plus cycloheximide-treated controls.

24 hr. As shown in Fig. 7, addition of 0.35 μM cycloheximide abolished the T_3-induced suppression but not the GH-induced suppression of both P450IIB1 and P450IIB2.

Discussion

Although both thyroidectomy and hypophysectomy caused an increase in hepatic P450IIB1 content, the level was 13–28-fold higher in hypophysectomized than in thyroidectomized rats. These results indicate the major role of pituitary factor on the suppression of P450IIB1 in rat livers. Consistent with our previous papers (18, 19), the hepatic level of P450IIB1 was influenced by pituitary GH (Table 3). The microsomal content was reduced by intermittent injection and by continuous infusion to a respective 49–65% and 5–8% of that in hypophysectomized rats. In addition, human GH suppressed the phenobarbital-induced level of P450IIB1 in primary cultured hepatocytes (Fig. 3) (48). These results indicate the principal role of pituitary GH on the suppression of hepatic P450IIB1, although T_3 exhibited a suppressive effect on P450IIB1 in hypophysectomized rats and in rat hepatocytes.

In contrast to the effect on P450IIB1, thyroidectomy was equally effective, compared with hypophysectomy, in the increase in P450IIB2 level in male and female rats. In addition, the increased levels were effectively reversed by treatment of thyroidectomized or hypophysectomized rats with T_3. These results indicate the direct involvement of thyroid hormone on the suppression of P450IIB2 in rat livers. As reported previously (18), the microsomal content of P450IIB2 was also reduced by treatment of hypophysectomized rats with GH. In addition, human GH suppressed P450IIB2 in hepatocytes (Fig. 3). These results suggest the suppressive role of pituitary GH. However, the sex-related difference was observed only for P-450IIB1 and not for P450IIB2 in intact rats, in spite of the sex-associated difference in secretion profile of GH in this species. Furthermore, treatment of rats with propylthiouracil, which decreased plasma level of thyroid hormone, also caused a pronounced increase of P450IIB2. This treatment has been shown to increase or decrease the plasma level of GH, depending on the period of administration, but does not severely deplete the plasma GH in rats (49). In addition, suppression of P450IIB2 occurred at physiological concentrations of thyroid hormones. Thus, these results favor the idea that thyroid hormone, rather than pituitary GH, has the dominant role in suppressive regulation of P450IIB2 in rat livers.

As shown in Fig. 4, T_4 and D-T_3 showed similar inhibition of both P450IIB1 and P450IIB2. T_3-Ac acid showed slightly less potent suppression, whereas r-T_3 had no clear effect of P450IIB1 and P450IIB2 levels. These results are largely consistent with the relative potencies of the analogues for thyroid hormone action *in vivo*. A recent study suggests that thyroid hormone activates liver malic enzyme through interaction at the thyroid hormone-responsive region located in the 5′-flanking region of the malic enzyme gene (50). Although the functional significance remains to be determined, it is interesting to note that a sequence (AGGGGNNGA) showing similarity to the thyroid hormone-responsive element of GH and malic enzyme genes exists upstream of a modified TATA sequence, CATAAA, in the 5′-flanking region reported for P450IIB1 and P450IIB2 (51, 52).

As indicated in Fig. 6, maximum suppression of P450IIB1 and P450IIB2 induced by T_3 was observed only when T_3 was added before the addition of phenobarbital, and late addition of T_3 instead enhanced the hepatic levels in rat hepatocytes. In the present study, treatment of propylthiouracil-pretreated rats with T_3, together with phenobarbital, decreased the hepatic level of P450IIB1/IIB2 mRNA, compared with the controls treated only with phenobarbital (data not shown). As shown in Fig. 5, T_3 suppressed the level of hepatic P450IIB mRNA. These results indicate that thyroid hormone suppresses hepatic P450IIB1 and P450IIB2 mainly by interacting at a pretranslational step of their syntheses in both phenobarbital-induced and constitutive states. In the present study, the level of P450IIB mRNA was suppressed by GH, which is consistent with data recently reported (48). However, some differences were observed between the effects of GH and thyroid hormone on the suppression of P450IIB1/IIB2; GH suppressed the induction of P450IIB1 and P450IIB2 by simultaneous addition with phenobarbital, whereas prior addition of T_3 is required for suppression. The reason for the difference is unclear, but it may be caused partly by the difference in the periods required for the recovery of thyroid hormone and GH receptors in hepatocytes (53, 54). However, addition of 0.35 μM cycloheximide abolished the T_3-induced suppression but not the GH-induced suppression of both P450IIB1 and P450IIB2 (Fig. 7). Thus, T_3 may also possibly mediate the suppression of P450IIB1 and P450IIB2 indirectly through a newly synthesized protein. In conclusion, the present study indicates that, in spite of a high level of similarity in their primary structures and drug-induced responses, P450IIB1 and P450IIB2 differ in susceptibility to thyroid hormone and GH, suggesting that P450IIB2 is under the suppressive influence of thyroid hormone in rat liver.

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