DECREASE IN SERUM THYROXINE LEVEL BY PHENOBARBITAL IN RATS IS NOT NECESSARILY DEPENDENT ON INCREASE IN HEPATIC UDP-GLUCURONOSYLTRANSFERASE

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ABSTRACT:

We have previously reported that there is a poor correlation between increase in the levels of UDP-glucuronosyltransferases, UGT1A1 and UGT1A6, and decrease in the levels of serum total thyroxine (T₄) and free T₄ in phenobarbital (PB)-treated rats, although the PB-induced decrease in rats is generally thought to occur through induction of the UDP-glucuronosyltransferase (T₄-UDP-GT: UGT1A1 and UGT1A6). In the present study, to clarify a relationship between the decrease in serum T₄ level and the increase in the T₄-UDP-GT activity by PB in rats, we examined the relationship using Gunn rats, a mutant strain of Wistar rats deficient in UGT1A isomers. Levels of serum total T₄, free T₄, and total triiodothyronine (T₃) were markedly decreased not only in Wistar rats but also in Gunn rats 1 day after the final administration of PB (80 mg/kg i.p., once daily for 4 days), and no significant difference in magnitude of the decrease between Wistar and Gunn rats was observed. On the other hand, the level and activity of T₄-UDP-GT were significantly increased by treatment with PB in Wistar rats but not in Gunn rats. Furthermore, significant decrease in the activity of hepatic type I iodothyronine deiodinase, which mediates the deiodination of T₄ and T₃, by PB treatment was observed in both Wistar and Gunn rats. In addition, no significant change in the level of serum thyroid-stimulating hormone, the activity of hepatic sulfotransferase, and the binding of [¹²⁵I]T₂ to serum transthyretin and albumin by PB treatment was observed in either Wistar or Gunn rats. In conclusion, the present results demonstrate that the decrease in serum total T₄ level by PB in Gunn rats is not dependent on the increase in hepatic T₄-UDP-GT activity and suggest that even in Wistar rats, the PB-induced decrease in serum T₄ level does not occur only through increase in hepatic T₄-UDP-GT.

Phenobarbital (PB) is well known to decrease the level of serum thyroid hormone and to increase the activities of hepatic drug-metabolizing enzymes in rats and mice (O’Connor et al., 1999; Hood et al., 2003). Furthermore, PB increases levels of serum thyroid-stimulating hormone (TSH) and thyroid gland growth in rats (Hood et al., 1999). As a possible mechanism for the PB-induced decrease in the level of serum thyroid hormone, enhancement of thyroid hormone metabolism by PB is considered (McClain, 1989; Capen, 1997). Especially, the decrease in the level of serum thyroxine (T₄) by PB in rats is thought to occur mainly through the induction of T₄-UDP-glucuronosyltransferase (T₄-UDP-GT), responsible for glucuronidation of T₄ (Barter and Klaassen, 1992a; Liu et al., 1995). This hypothesis is supported by the previous reports that a number of T₄-UDP-GT inducers, such as polychlorinated biphenyl (PCB), 3-methylcholanthrene, and pregnenolone-16α-carbonitrite, show the ability to decrease serum thyroid hormone (Saito et al., 1991; De Sandro et al., 1992; Barter and Klaassen, 1994). However, the magnitude of decrease in the level of serum total T₄ by PB is not necessarily correlated with that of increase in T₄-UDP-GT activity (Saito et al., 1991; Hood et al., 2003). Likewise, our preliminary study (Suzuki et al., 2004) has indicated that there is a poor correlation between increase in the levels of UGT1A1 and UGT1A6 and decrease in the levels of serum total T₄ and free T₄ in PB-treated rats.

In the present study, therefore, we examined a relationship between the decrease in serum total T₄ level and the increase in hepatic T₄-UDP-GT (UGT1A1 and UGT1A6) by PB using UGT1A-deficient Wistar rats (Gunn rats) and demonstrated that the PB-induced decrease in serum total T₄ level in rats was not necessarily dependent on the increase in hepatic T₄-UDP-GT activity.

Materials and Methods

Chemicals. PB was purchased from Nakakita Yakuhin Co., Ltd. (Aichi, Japan). The [¹²⁵I]-reverse triiodothyronine (T₃) and [¹³¹I]T₂, radiolabeled at the 5'-position of the outer ring, was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). All other chemicals were obtained commercially in appropriate grades of purity.

Animal Treatments. Male Wistar rats (160–200 g) and UGT1A-deficient Wistar rats (Gunn rats, 190–260 g) were obtained from Japan SLC., Inc.
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(Shizuoka, Japan). Male Wistar and Gunn rats were housed three or four per cage with free access to commercial chow and tap water, maintained on a 12-h dark/light cycle (8:00 AM-8:00 PM light) in an air-controlled room (temperature, 24.5 ± 1°C; humidity, 55 ± 5%), and handled with humane care under the guidelines of the University of Shizuoka (Shizuoka, Japan). Rats received four consecutive intraperitoneal injections of PB (80 mg/kg) dissolved in 0.9% saline (5 ml/kg). Control animals were treated with a vehicle alone (5 ml/kg). All rats were killed by decapitation 1 day after the final administration. The liver was removed, and hepatic microsomes and cytosols were prepared according to the method of Kato et al. (1995) and stored at −85°C until used. Blood was collected from each animal between 10:30 and 11:30 AM. After clotting at room temperature, serum was separated by centrifugation and stored at −50°C until used.

**Analysis of Serum Hormones.** Levels of serum total $T_4$ (free $T_4$, total $T_3$, and TSH were measured by radioimmunoassay using the Total T4 and Free T4 kit (Diagnostic Products Corporation, Los Angeles, CA), the $T_3·$RIA BEAD (Abbott Japan Co., Ltd., Tokyo, Japan), and the rTSH $^{[125I]}$ Biotrak assay system (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK), respectively.

**Hepatic $T_4$ Metabolizing Enzyme Assays.** Amounts of proteins of hepatic subcellular fractions, microsomes and cytosols, were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The activity of microsomal UDP-GT toward $T_4$ was determined by the method of Barter and Klaassen (1992b). The activity of microsomal type I iodothyronine deiodinase (ID-I) was determined by the method of Hood and Klaassen (2000). The activity of cytosolic sulfotransferase toward $T_4$ was determined by the method of Kaptein et al. (1997).

**Western Blot Analysis.** Polyclonal anti-peptide antibodies against the common region of UGT1A isoforms and specific antibodies against UGT1A1, UGT1A6, and UGT2B1 were used (Ishikeshi et al., 1995, 1997). Western blot analyses for microsomal UGT isoforms were performed by the method of Luquita et al. (2001). Isolated proteins corresponding to UGT1A1, UGT1A6, and UGT2B1 were detected using chemical luminescence (ECL detection kit; Amersham Biosciences Inc., Piscataway, NJ), and the level of each protein was determined densitometrically with LAS-1000 (Fuji Photo Film Co., Ltd., Tokyo, Japan).

**Analysis of $^{[125I]}T_4$ Binding to Serum Proteins.** At 1 day after a consecutive 4-day treatment with PB, rats were anesthetized with 50 mg/ml sodium pentobarbital combined 1:1 with 1 mg/ml potassium iodide at 2 mg/ml. The femoral artery was cannulated (polyethylene tube SP31; Natsume Inc., Tokyo, Japan) and primed with heparinized saline (33 units/ml). Fifteen minutes later, rats were given 1 ml of $^{[125I]}T_4$ i.v., at 15 μCi/ml in 10 mM NaOH saline + 1% normal rat serum. After the administration of $^{[125I]}T_4$, a portion (0.5 ml) of blood was sampled from the artery at the indicated time, and serum was collected and stored at −50°C for assay. Serum was diluted in 100 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM diithiothreitol, and 30% glycerol, and subjected to electrophoresis on 4 to 20% gradient native polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue R-250 (Amersham Pharmacia Biotech, Uppsala, Sweden) and dried for 20 h at room temperature. The dried gels were radioautographed for 20 h at room temperature using Imaging Plate 2040 (Fuji Photo Film Co., Ltd.). The levels of $^{[125I]}T_4$-albumin and $^{[125I]}T_4$-TTR complexes were determined by counting the gel fractions identified from a Bio Imaging Analyzer (BAS-2000II IP Reader; Fuji Photo Film Co., Ltd.).

**Statistics.** The data obtained were statistically analyzed according to Dunnett’s test after the analysis of variance.

**Results**

**Serum Hormone Levels.** Constitutive levels of serum total $T_4$ (free $T_4$, total $T_3$, and TSH were more than 1.8-fold higher in Gunn rats than in Wistar rats. Effects of PB on levels of serum thyroid hormones were next examined in Wistar and Gunn rats (Fig. 1). In both Wistar and Gunn rats, PB treatment resulted in decreases of serum total $T_4$, free $T_4$, and total $T_3$, and the magnitude of the decrease in each serum thyroid hormone was almost the same in both rats. On the other hand, no significant change in the level of serum TSH by PB treatment was observed in either Wistar or Gunn rats.

**Hepatic $T_4$ Metabolizing Enzyme Activities.** It has been reported that $T_4$ glucuronidation is primarily mediated by UGT1A enzymes, UGT1A1 and UGT1A6, in the rat liver (Visser, 1996). Therefore, we examined effects of PB on hepatic microsomal $T_4$-UDP-GT activity in Wistar and Gunn rats. Constitutive activity of $T_4$-UDP-GT was more than 2.1-fold higher in Wistar rats than in Gunn rats. PB treatment resulted in a significant increase of $T_4$-UDP-GT activity in Wistar rats but not in Gunn rats (Fig. 2).

Hepatic microsomal ID-I activity in both Wistar and Gunn rats was significantly decreased by PB (Fig. 3). On the other hand, no significant change in activity of hepatic sulfotransferase by PB treatment was observed in either PB-treated Wistar or Gunn rats (data not shown).
Immunoblot Analysis for UGT1As. Levels of immunoreactive proteins responsible for UGT1A isoforms, UGT1A1 and UGT1A6, were increased by PB in Wistar rats but not in Gunn rats (Figs. 4 and 5). In addition, no constitutive expression of the UGT1A isoforms was confirmed in Gunn rats. On the other hand, the level of UGT2B1 was significantly increased by PB in both Wistar and Gunn rats, and the magnitude of the increase was higher in Gunn rats than in Wistar rats (Figs. 4 and 5).

Serum Protein Binding of [125I]T4. The effect of PB on the binding of [125I]T4 to serum proteins, TTR and albumin, was examined in Wistar rats. No significant change in the binding level of [125I]T4 to each serum protein by PB treatment was observed with the exception of a decrease in the level of [125I]T4-TTR complex at 120 min after [125I]T4 administration (Fig. 6).

Discussion
In the present study, we found that treatment with PB resulted in a drastic decrease in serum total T4 and free T4 levels in both Wistar and Gunn rats, although significant increase in the activity of T4-UDP-GT occurred only in Wistar rats and not in Gunn rats. The present findings demonstrate that in Gunn rats, PB-induced decrease in the level of serum T4 occurs in a hepatic T4-UDP-GT-independent fashion. In addition, constitutive levels of serum total T4 and T3 were more than 1.8-fold higher in Gunn rats than in Wistar rats, suggesting that the deficit of T4 glucuronidation results in higher T4 serum levels. Similar results and suggestions have been obtained by Benathan et al. (1983).

In general, T4-UDP-GT inducers, including PB, clobazam, PCB, 3-methylcholanthrene, and pregnenolone-16α-carbonitrile, have been considered to decrease a level of serum T4 through an increase in hepatic T4-UDP-GT (Barter and Klaassen, 1994; Van Birgelen et al., 1995; Miyawaki et al., 2003). However, it has been reported that the difference between rats and mice in the magnitude of decrease in the level of serum total T4 by an inducer of T4-UDP-GT is not well...
hydroxylated metabolites to TTR, a major T₄-transporting protein, might be attributed, in part, to a decrease in the level of T₄ in PCB-treated rats (Lans et al., 1993; Brouwer et al., 1998; Kato et al., 2004), displacement of T₄ from serum TTR by PB did not occur in PB-treated rats, with the exception of the slight displacement in Wistar rats at 120 min after PB treatment. Accordingly, the decrease in the level of T₄ in PB-treated rats occurs in a TTR-independent pathway. In addition, no change in activity of hepatic sulfotransferase, which efficiently catalyzes the sulfation of iodothyronines (Kester et al., 1999), by PB treatment was observed in either Wistar or Gunn rats.

In conclusion, the present findings demonstrate that the decrease in serum total T₄ level by PB in Gunn rats occurs without increases in hepatic T₄-metabolizing enzymes (T₄-UDP-GT, ID-I, and sulfotransferase), the binding of PB to serum TTR, and the level of serum TSH, although an exact mechanism for the PB-induced decrease remains unclear. In Wistar rats, PB-induced T₄-UDP-GT might also contribute, in part, to the decrease in serum T₄ level. To clarify the exact mechanisms for the PB-induced decrease in serum thyroid hormones, further studies involving T₄ transporters, nonhepatic T₄-UDP-GT, and exchangeable thyroid hormone pools in nonhepatic tissues would be necessary.

References


FIG. 6. Effect of PB on the binding of [125I]T₄ to the serum proteins in Wistar rats. The binding of [125I]T₄ to the serum proteins was assessed by nondenaturing polyacrylamide gel electrophoresis, as described under Materials and Methods. Each column represents the mean ± S.E. (vertical bars) for three animals. * P < 0.05, significantly different from each control.


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