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The effects of clobazam treatment in rats on the expression of genes and proteins encoding glucronosyltransferase 1A/2B (UGT1A/2B) and multidrug resistance-associated protein-2 (MRP2), and development of thyroid follicular cell hypertrophy

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ABSTRACT

Clobazam (CLB) is known to increase hepatobiliary thyroxine (T4) clearance in Sprague–Dawley (SD) rats, which results in hypothyroidism followed by thyroid follicular cell hypertrophy. However, the mechanism of the acceleration of T4-clearance has not been fully investigated.

In the present study, we tried to clarify the roles of hepatic UDP-glucronosyltransferase (UGT) isoenzymes (UGT1A and UGT2B) and efflux transporter (multidrug resistance–associated protein-2; MRP2) in the CLB-induced acceleration of T4-clearance using two mutant rat strains, UGT1A-deficient mutant (Gunn) and MRP2-deficient mutant (EHBR) rats, especially focusing on thyroid morphology, levels of circulating hormones (T4 and triiodothyronine (T3)) and thyroid-stimulating hormone (TSH), and mRNA or protein expressions of UGTs (Ugt1a1, Ugt1a6, and Ugt2b1/2) and MRP2 (Mrp).

CLB induced thyroid morphological changes with increases in TSH in SD and Gunn rats, but not in EHBR rats. T4 was slightly decreased in SD and Gunn rats, and T3 was decreased in Gunn rats, whereas these hormones were maintained in EHBR rats. Hepatic Ugt1a1, Ugt1a6, Ugt2b1/2, and Mrp2 mRNAs were upregulated in SD rats. In Gunn rats, UGT1A mRNAs (Ugt1a1/6) and protein levels were quite low, but UGT2B mRNAs (Ugt2b1/2) and protein were prominently upregulated. In SD and Gunn rats, MRP2 mRNA and protein were upregulated to the same degree. These results suggest that MRP2 is an important contributor in development of the thyroid cellular hypertrophy in CLB-treated rats, and that UGT1A and UGT2B work in concert with MRP2 in the presence of MRP2 function to enable the effective elimination of thyroid hormones.

Introduction

Some chemicals exert a direct action on the thyroid gland by disrupting one or more of the steps involved in the biosynthesis and secretion of thyroid hormones. Such thyroid disruption may occur via several mechanisms, including (1) disruption of thyroid hormone synthesis via inhibition of thyroperoxidase (e.g., propylthiouracil) (Takayama et al., 1986) or iodide uptake (e.g., perchlorate (ClO_4^-) and thiocyanate (SCN^-)) (Kanno et al., 1990; Mannisto et al., 1979); (2) prevention of organic binding of iodine and coupling of T4 and T3 (e.g., thiourea and propylthiouracil) (Kanno et al., 1990; Mannisto et al., 1979); and (3) inhibition of thyroid hormone secretion through an effect on proteolysis of active thyroid hormone from the colloid (e.g., lithium) (Radvila et al., 1976).

In addition to the above intrathyroidal mechanisms, the following mechanisms of action have been reported recently: suppression of iodide transport via competition or inhibition of sodium iodide symporter (NIS) (e.g., perchlorate (ClO_4^-)) (Van Sande et al., 2003), binding to thyroid hormone transport protein transthyretin (TTR) (e.g., polychlorinated biphenyl metabolites) (Kato et al., 2009), upregulation of iodothyronine deiodinases (e.g., octyl-methoxycinnamate (OMC)) (Klammer et al., 2007), and agonistic or antagonistic action of chemicals at the thyroid hormone receptor (e.g., bisphenol A) (Kitamura et al., 2005). On the other hand, some chemicals exert an indirect action on the thyroid gland by altering the thyroid hormone disposition by an extrathyroidal mechanism. This results from accelerated peripheral elimination of thyroid hormones in rats (e.g., phenobarbital (PB) (McClain et al., 1989),

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pregnenolone-16a-carbonitrile (PCN) (Liu et al., 1995), and a variety of polychlorinated biphenyls (PCBs) (Craft et al., 2002). These effects involve hepatic microsomal enzyme induction and increased glucuronidation of T4 to enhance hepatobiliary clearance of the conjugated hormone (Capen, 1994). Currently, induction of hepatic basolateral organic anion transporters is assumed to be directly associated with uptake of thyroid hormone (Saghir et al., 2008). Additionally, ATP-dependent transporters localized on the canalicular membranes in the liver are known to contribute to the biliary excretion (Klaassen and Lu, 2008; Lehman-McKeeman, 2008). Attention has been especially focused on the role of MRP2 in the transportation of conjugated organic anions into the bile (Leslie et al., 2005; Nies and Keppler, 2007). Recently, some chemicals have been reported to induce hepatic MRP2 proteins, suggesting an association with the hepatic and systemic disposition of thyroid hormones (Szabo et al., 2009; Yu et al., 2011).

CLB is an antiepileptic drug that exerts its effect by binding specifically to CNS benzodiazepine receptors (Nakajima, 2001). It has been widely used as a long-term adjunctive therapy with other antiepileptic agents (Remy, 1994). In non-clinical safety studies, CLB is known to induce thyroid tumor in rats, due to an extrathyroidal system via an increase in the elimination of circulating thyroid hormones (Miyawaki et al., 2003). In addition, CLB causes hepatomegaly in rats via Cyp2b1, Cyp3a1/3a2, and Ugt2b2 upregulations (Miyawaki et al., 2011). However, the contribution of UGT isoforms to the elimination of thyroid hormones from circulation by CLB is not fully determined, nor is that of hepatic efflux transporters.

Thus, in the present study, we tried to clarify the roles of UGT isoenzymes (UGT1A, 2B) and efflux transporter (MRP2) in development of the thyroid cellular hypertrophy caused by 2-week treatment with CLB using two mutant rat strains, UGT1A-deficient mutant (Gunn) and MRP2-deficient mutant (EHBR) rats, and the normal strain (SD rat), in light of morphology together with serum thyroid-associated hormone (T3, T4, and TSH) changes and hepatic UGT1A, UGT2B, and MRP2 mRNA and protein expressions.

Materials and methods

Chemicals. Clobazam (Lot number 24567; purity 99.8%) was supplied by Dainippon Sumitomo Pharm. Co., Ltd. (Osaka, Japan).

Animals and husbandry. Animal usage for the study was reviewed and approved by the Committee for the Ethical Usage of Experimental Animals of Dainippon Sumitomo Pharma Co., Ltd. Male Sprague–Dawley (Slc:SD), Gunn/Slc-j/j, and EHBR/Eis rats were purchased from Japan SLC, Inc. (Shizuoka, Japan), respectively. After a week of acclimation, healthy rats at 5 weeks of age weighing 157.5–196.8 g for SD, 150.3–198.6 g for EHBR, and 101.1–129.4 g for Gunn strains were used in the study. The animals were housed individually in a barrier-sustained room with controlled temperature of 24 °C ± 2 °C and relative humidity of 55% ± 10% and a 12-h light (8 a.m. – 8 p.m.)/dark cycle. The rats were given access to autoclaved, pelleted food (CRF-1; Oriental Yeast Co., ltd. Japan) and tap water ad libitum.

Animal study design. Five rats per group per strain were randomly assigned to control and CLB groups as shown in Table 1. We have previously reported that a 400 mg/kg/day of CLB treatment for 28-day caused thyroid follicular hypertrophy in male SD rats (Miyawaki et al., 2003). A 400 mg/kg was set as the dose in this study in order to compare the difference of CLB-induced hypothyroid effect among SD, Gunn and EHBR rats. Powdered potato starch, used as a vehicle, was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CLB was suspended in a 2% (w/v) aqueous solution of potato starch, with a final CLB concentration of 8% (w/v). Dosing formulation volumes were calculated based on the latest body weights of animals (5 mL/kg). The dosing formulations were drawn into polypropylene syringes while being stirred using a magnetic stirrer, and were promptly administered orally via intubation once a day for 2 weeks. Animals in the control group of each strain were given the same volume of the 2% starch solution alone in the same manner. Observation for clinical signs and mortality was conducted daily, and body weight was measured twice a week throughout the dosing period in order to check the health condition. On the day after final administration, the blood was collected from anesthetized rats, which were then euthanized and necropsied. The collected blood samples and tissue samples (liver and/or thyroid glands) were used for analysis of serum TSH, total T4, and total T3 concentrations, and histopathological examination respectively. In addition, a part of the liver tissue was used for gene and protein analyses.

Table 1
Study design.
Control: 2% (w/v) aqueous solution of potato starch.

Drug	Dosage (mg/kg)	Number of animals		
		SD	EHBR	Gunn
Control	0	5	5	5
CLB	400	5	5	5

Determination of serum TSH and thyroid hormone. Five milliliter aliquots of blood were drawn from the caudal vena cava of animals. Serum was separated by centrifugation (4 °C, 1,800 × *g*, 10 min) and TSH, total T4 and total T3 concentrations were measured the following methods. Serum T3 and T4 concentrations were measured using Access immunoassay system, Access 2 (Beckman Coulter Inc.). The standard curve for T3 or T4 was constructed by plotting luminescence of each reference standard at 540 nm, and T3 or T4 concentration in each test sample was calculated from each standard curve. Serum TSH concentration was measured using a commercial ELISA kit, Rodent TSH ELISA Test Kit (Endocrine Technologies Inc., Newark, CA). A standard curve was constructed by plotting the absorbance of the reference standards at 450 nm, and TSH concentration in each test sample was calculated from the standard curve.

Histopathological examination. On the day after 2 weeks of treatment, the animals were euthanized by exsanguinations following blood sample collection under isoflurane anesthesia, and were necropsied at 10 a.m. The liver and thyroid gland (right side) were immediately removed and weighed, and a relative organ weight (g/100 g BW or mg/100 g BW) was calculated using the body weight recorded on the day of necropsy. These operations were completed within 0.5 h. A part of the liver and whole thyroid glands from each animal were fixed in 10% neutral buffered formalin, routinely processed for embedding in paraffin wax, sectioned, and stained with hematoxylin and eosin (HE) for microscopic examination. In addition to the HE staining, immunohistochemical staining was applied for examination of the cell proliferation in the thyroid glands. The cell proliferation examination was performed in accordance with standard procedures using a mouse monoclonal anti-rat Ki-67 primary antibody (clone: MIB-5, Dako Japan Inc., Japan). However, no cell proliferation was seen in the thyroid glands of all rats (data not shown).

Extraction of total RNA. A part of the liver from each animal was stored in QIAGEN RNAlater (QIAGEN K.K., Tokyo, Japan), RNA stabilization reagent, at −20 °C until use. Total RNA was extracted from the liver using QIAGEN RNeasy mini kits and eluted in 20 μl RNase-free water.

Real-time RT-PCR analysis. Quantitative gene expression levels were determined using real-time PCR with the ABI Prism 7500 (Applied Biosystems, Foster City, CA) and TaqMan probes (FAM and TAMRA dyes labeled). Primers shown in Table 2 were created using Applied