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Disruption of thyroid hormone homeostasis in Ugt1a-deficient Gunn rats by microsomal enzyme inducers is not due to enhanced thyroxine glucuronidation☆

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Abstract

Microsomal enzyme inducers (MEI) that increase UDP-glucuronosyltransferases (UGTs) are thought to increase glucuronidation of thyroxine (T₄), thus reducing serum T₄, and subsequently increasing thyroid stimulating hormone (TSH). Ugt1a1 and Ugt1a6 mediate T₄ glucuronidation. Therefore, this experiment determined the involvement of Ugt1a enzymes in increased T₄ glucuronidation, decreased serum T₄, and increased TSH after MEI treatment. Male Wistar and Ugt1a-deficient Wistar (Gunn) rats were fed a control diet or diet containing pregnenolone-16 α -carbonitrile (PCN; 800 ppm), 3-methylcholanthrene (3-MC; 200 ppm), or Aroclor 1254 (PCB; 100 ppm) for 7 days. Serum T₄, triiodothyronine (T₃), and TSH concentrations, hepatic T₄/T₃ glucuronidation, and thyroid histology and follicular cell proliferation were investigated. PCN, 3-MC, and PCB treatments decreased serum T₄, whereas serum T₃ was maintained in both Gunn and Wistar rats (except for PCB treatment). TSH was increased in Wistar and Gunn rats after PCN (130 and 277%) or PCB treatment (72 and 60%). T₄ glucuronidation in Wistar rats was increased after PCN (298%), 3-MC (85%), and PCB (450%), but was extremely low in Gunn rats, and unchanged after MEI. T₃ glucuronidation was increased after PCN (121%) or PCB (58%) in Wistar rats, but only PCN increased T₃ glucuronidation in Gunn rats (43%). PCN treatment induced thyroid morphological changes and increased follicular cell proliferation in both strains. These data demonstrate that T₄ glucuronidation cannot be increased in Ugt1a-deficient Gunn rats. Thus, the decrease in serum T₄, increase in TSH, and increase in thyroid cell proliferation after MEI are not dependent on increased T₄ glucuronidation, and cannot be attributed to Ugt1a enzymes.

Keywords

Phenobarbital; Pregnenolone-16 α -carbonitrile; 3-methylcholanthrene; Aroclor 1254; Thyroxine T₄; Thyroid stimulating hormone; UDP-glucuronosyltransferases; Ugt1a-deficient Gunn rat

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Conflict of interest statement

The authors do not have any conflict of interest.

Introduction

Hepatic microsomal enzymes are important in the maintenance of thyroid hormone (TH) homeostasis. Xenobiotic induction of these enzymes is thought to lower serum TH concentrations by increasing TH glucuronidation and excretion into bile and urine (Klaassen and Hood, 2001; Kato et al., 2008). Increased glucuronidation and sulfation, two major metabolic pathways in the elimination of thyroxine (T_4) and triiodothyronine (T_3), can disrupt the hypothalamic–pituitary–thyroid axis. Decreased TH concentrations in blood can lead to increased secretion of TSH from the pituitary to compensate for the changes in circulating hormone concentrations. Excessive TSH stimulation can cause changes in thyroid follicular cells, including hypertrophy (increase in cell size) and hyperplasia (increase in cell number), ultimately leading to tumor formation (McClain, 1992; Capen, 2008).

Phenobarbital (PB), a prototypical microsomal enzyme inducer (MEI), has been studied extensively for its role in thyroid tumor promotion. PB exposure increases thyroid gland weight, causes follicular cell hypertrophy and hyperplasia, and increases biliary excretion of T_4 (McClain et al., 1988, 1989; Meek et al., 2003). PB and other MEI, such as pregnenolone-16 α -carbonitrile (PCN), 3-methyl-cholanthrene (3-MC), and Aroclor 1254 (PCB), induce UDP-glucuronosyltransferases (Ugts) in addition to cytochrome P450s. Several investigators have studied the effects of MEI on serum T_4 concentration. PB, PCN, 3-MC, and PCB reduce serum T_4 (McClain et al., 1989; Barter and Klaassen, 1994; Liu et al., 1995). These effects occur through an extrathyroidal mechanism (Barter and Klaassen, 1992), supposedly through increased hepatic glucuronidation of T_4 (both *in vitro* and *in vivo*) (Barter and Klaassen, 1992; Liu et al., 1995), and increased *in vivo* biliary excretion of T_4 -glucuronide (Japundzic et al., 1976; Collins and Capen, 1980; Vansell and Klaassen, 2001).

Biotransformation pathways for thyroid hormone metabolism include glucuronidation, sulfation, and deiodination by Ugts, sulfotransferases, or deiodinases, respectively (Kohrle et al., 1991; Curran and Degroot, 1991). Ugts catalyze the formation of glucuronide conjugates by adding glucuronic acid to molecules with hydroxyl, amino, carboxy, or mercapto groups. Ugts in rodents have been divided into two major families, Ugt1 and Ugt2, based on their gene sequence and identity. The Ugt1 family of enzymes arises from a single gene, whereas the Ugt2 family members are products of distinct, individual genes (Emi et al., 1995; Clarke and Burchell, 1997; Ritter, 2000). Research indicates the involvement of the Ugt1a family of enzymes that glucuronidate T_4 , namely Ugt1a1 (bilirubin) and Ugt1a6 (phenol). Hepatic T_4 glucuronidation is impaired in Gunn rats, a mutant strain of Wistar rats deficient in all *Ugt1a* enzymes (Kato et al., 2004, 2005, 2007). However, T_3 glucuronidation is unaffected in Gunn rats (Beetstra et al., 1991; Visser et al., 1993; Kato et al., 2004). Thus, it appears that T_4 is primarily glucuronidated by Ugt1a enzymes, whereas T_3 is glucuronidated by Ugt2b enzymes, presumably Ugt2b2 (androsterone) (Beetstra et al., 1991; Visser et al., 1993).

The mechanism by which MEI(s) increase TSH has not been clearly elucidated. Treatment of rats with PCN and PB resulted in increases in serum TSH, whereas treatment with 3-MC and PCB did not (Liu et al., 1995; Hood et al., 1999; Vansell and Klaassen, 2001). Interestingly, all of these inducer treatments are effective at increasing the glucuronidation of T_4 and decreasing serum T_4 , yet have different effects on serum TSH. Unlike serum T_4 , serum T_3 concentrations are not markedly decreased by enzyme inducers, and are relatively maintained (Liu et al., 1995; Hood et al., 1999). Previous studies indicate inducers that increased T_3 glucuronidation (PCN and PB) also increased serum TSH, whereas treatment with 3-MC and PCB did not (Hood and Klaassen, 2000a).

Whereas it is generally thought that the increase in thyroid cell proliferation and tumors after treatment with MEI is due to an increase in TSH subsequent to increased glucuronidation of T₄ (McClain et al., 1989; Capen, 2008), the data suggests that there is not a good correlation with the ability of MEI to increase T₄ glucuronidation and to increase TSH (Hood and Klaassen, 2000a; Vansell and Klaassen, 2001). Therefore, the purpose of the present study was to determine whether the increased T₄ glucuronidation after treatment with MEI is due to the Ugt1a family of enzymes, using Ugt1a-deficient Gunn rats as a model. We hypothesized that the Ugt1a-deficient Gunn rats cannot glucuronidate T₄ as compared to its parent strain of Wistar rats. Whether the decrease in serum T₄ produced by MEI is dependent on Ugt1a enzymes, and whether the increase in TSH produced by MEI is dependent upon Ugt1a enzymes were also examined.

Materials and methods

Chemicals

Pregnenolone-16 α -carbonitrile (PCN), 3-methylcholanthrene (3-MC), thyroxine (T₄), triiodothyronine (T₃), and uridine diphosphoglucuronic acid (UDP-GA) were obtained from Sigma Chemical (St. Louis, MO). Aroclor 1254 (PCB) was generously provided by Dr. Larry Hansen (University of Illinois, Urbana, IL). Sephadex LH-20 bed resin and polystyrene columns were obtained from Amersham Biosciences (Piscataway, NJ), and Pierce Chemical (Rockford, IL), respectively. ¹²⁵I-T₄ and ¹²⁵I-T₃ (150 μ Ci/ μ g) were each obtained from Perkin Elmer Life Sciences (New England Nuclear, Boston, MA). Radioimmunoassay (RIA) kits for total and free T₄ and T₃, and rat thyroid stimulating hormone (TSH) were obtained from Diagnostics Product Corporation (Los Angeles, CA) and Amersham Biosciences (Piscataway, NJ), respectively. Anti-proliferating cell nuclear antigen (PCNA) was obtained from Zymed, Inc. (San Francisco, CA). Biotinylated anti-mouse IgG (rat adsorbed) and the ABC Elite kit were obtained from Vector Labs (Burlingame, CA). Diaminobenzidine (DAB) and Histomark Black were obtained from Kirkegaard and Perry (Gaithersburg, MD). All other reagents were obtained from Fisher Chemical (Pittsburgh, PA).

Animals and treatments

Male Wistar (*JJ*) and Gunn (*jj*) rats, 150–200 g, were obtained from Harlan (Indianapolis, IN) at approximately 60 days of age. Rats were individually housed in polypropylene cages containing corn-cob bedding and maintained at 22 $\pm$ 2 °C with a 12:12 h light:dark cycle in an AAALAC-accredited facility. PCN, 3-MC, and PCB were each dissolved in acetone, and each was added to 1 kg of Purina Rodent Chow (Harlan Teklad 8604) pellets to provide final concentrations in the feed of 800, 200, and 100 ppm, respectively. Control (acetone-treated) and PCN-, 3-MC-, and PCB-treated rat chow pellets were allowed to dry overnight. Rats were given the diets for 7 days as previously described (Hood and Klaassen, 2000a,b; Richardson and Klaassen, 2010). Rats were monitored daily and individual body weight and food consumption recorded. Liver, thyroid, and blood were collected on experimental day 8. Livers were weighed, frozen in liquid nitrogen, and stored at –80 °C until microsome preparation. Thyroid glands were removed, weighed, fixed in 4% paraformaldehyde, and stored at 4 °C prior to routine embedding in paraffin blocks and sectioning for slides. Blood was allowed to clot on ice, centrifuged at 5,000g for 20 min, and serum stored at –20 °C.

Microsome preparation

Liver microsomes were prepared by differential centrifugation as described by Hood and Klaassen (2000a). Pellets were stored at –80 °C, and resuspended in 0.25 M sucrose before assay. Protein concentration in liver microsomes was determined using BioRad DC Protein assay kit (Hercules, CA).

Determination of total and free T₄, T₃, and TSH serum concentrations

The concentrations of total (free and protein-bound) and free T₄, total and free T₃, and TSH were determined by radioimmunoassay. Limits of detection for each kit were 0.25 µg/dL, 0.01 ng/dL, 7 ng/dL, 0.2 pg/ml, and 0.5 ng/ml, respectively.

Purification of ¹²⁵I-T₄ and ¹²⁵I-T₃

Stock radioactive compounds were purified prior to each glucuronidation assay (Hood and Klaassen, 2000a). Briefly, aliquots of stock ¹²⁵I-T₄ and ¹²⁵I-T₃ were applied separately to a Sephadex LH-20 column (0.5 ml bed volume), equilibrated with 0.1 N HCl. Residual iodide was removed by rinsing the column with 4 ml of 0.1 N HCl, followed by rinsing with 4 ml deionized H₂O. ¹²⁵I-T₄ and ¹²⁵I-T₃ were eluted from the column by rinsing with 1.5 ml of 98% ethanol/2% NH₄OH solution. The volumes of ¹²⁵I-T₄ and ¹²⁵I-T₃ were reduced to ~ 500–600 µl under a stream of N₂ gas.

Glucuronidation of T₄ and T₃

Hepatic Ugt activities toward T₄ and T₃ were quantified as the amount of ¹²⁵I-T₄ or ¹²⁵I-T₃ glucuronide produced by the method of Beetstra et al. (1991), as modified by Hood and Klaassen (2000a). Duplicate and blank reaction mixtures (200 µl final volume) contained 75 mM Tris-HCl (pH 7.8), 7.5 mM MgCl₂, 30 mM UDP-GA, 0.1 mM propylthiouracil (to inhibit outer-ring deiodinases), 1 µM cold T₄ or T₃, and ¹²⁵I-T₄ or ¹²⁵I-T₃ (~100,000 cpm/reaction). UDP-GA solutions were made fresh before each assay. Reactions were initiated with the addition of microsomal protein (250 µg/ml for T₄, 80 µg/ml for T₃), and incubated for 60 min in a shaking waterbath at 37 °C. Blank reaction mixtures received 50 µl of 0.25 M sucrose instead of protein, and were also incubated for 60 min. To stop the reaction, 200 µl ice-cold 100% methanol was added to each vial, and the vials were placed on ice. Samples were centrifuged at 12,000 g (5 °C, 45 min), and 200 µl of each centrifuged sample was added to 800 µl 0.1 N HCl. Acidified samples were added to a Sephadex LH-20 column (1-ml bed volume) previously equilibrated with 0.1 N HCl. Free ¹²⁵I was eluted with 3 ml 0.1 N HCl. Columns were rinsed with aliquots of deionized H₂O (8 ml total) to elute ¹²⁵I-T₄ glucuronide or ¹²⁵I-T₃ glucuronide. Unconjugated ¹²⁵I-T₄ and ¹²⁵I-T₃ were eluted with 2 rinses of 3 ml ethanol-0.1 N NaOH (1:1, v:v). The amount of radioactivity in each sample was quantified using a Packard Model 5650 gamma spectrophotometer (Downers Grove, IL).

Proliferating cell nuclear antigen (PCNA) immunocytochemistry

Four micron sections of each thyroid were cut and mounted on polylysine coated slides. Slides were deparaffinized with xylenes and hydrated in a decreasing ethanol series and distilled water. For antigen retrieval, slides were microwaved at reduced power for 10 min in 1 mM citrate buffer (pH 6.0), and then allowed to cool (in buffer). Slides were rinsed in 0.1 M phosphate-buffered saline (PBS; pH 7.6) for 10 min and incubated in 3% H₂O₂ for 10 min. Slides were rinsed in PBS for 4 min, and incubated in 10% normal horse serum for 15 min. Following another PBS rinse, anti-PCNA antibody (prediluted manufacturer's solution was further diluted 1:10 with PBS) was added to the slides, and the slides were incubated overnight (18–24 h) at 4 °C, and then rinsed with PBS. Biotinylated anti-mouse IgG antibody (1:200) was added to the slides, incubated for 30 min, and then rinsed with PBS. The slides were incubated in avidin-biotin complex (ABC) solution (per manufacturer's instructions) for 30 min, rinsed in PBS, incubated with Histomark Black/DAB solution (3–5 min), and rinsed with deionized water.

Assessment of thyroid follicular cell proliferation

The labeling index (LI) was determined by counting the number of labeled nuclei, which were stained dark brown/black. Approximately 500–1000 nuclei (labeled plus unlabeled) of each thyroid gland section were counted by microscopic examination. To reduce bias during counting, slides and tissue sections were chosen at random. Both labeled and unlabeled thyroid follicular cell nuclei from each section were counted, until at least 500 cell nuclei were assessed.

Statistical analysis

Means and standard errors were calculated. Differences between control and treated groups were determined using a one-way ANOVA, followed by the posthoc Tukey's test ($p \leq 0.05$). Data are presented as mean $\pm$ SE, with 5–6 replications for each group. Statistical analyses were performed using STATISTICA 4.5, Statsoft, Inc. (Tulsa, OK).

Results

Effect of microsomal enzyme inducers on T₄ and T₃ glucuronidation

Glucuronidation of thyroxine (T₄) in livers of control Gunn rats was 51% lower than in Wistar rats (Fig. 1, top panel). Treatment with PCN, 3-MC, and PCB dramatically increased the glucuronidation of T₄ in Wistar rats by 298, 85, and 450%, respectively. In contrast, there was no significant change in glucuronidation of T₄ in Gunn rats regardless of inducer treatment. Glucuronidation of triiodothyronine (T₃) in control Gunn rats was 31% higher than in Wistar rats (Fig. 1, bottom panel). Treatment with PCN or PCB significantly increased glucuronidation of T₃ in Wistar rats by 121 and 58%, respectively, with no apparent effect with 3-MC treatment. However, PCN was the only inducer to significantly increase T₃ glucuronidation (43%) in Gunn rats.

Effect of microsomal enzyme inducers on serum T₄ and T₃

Total serum T₄ in control Gunn rats was 21% higher than in Wistar rats (Fig. 2, top panel). Treatment of Wistar rats with PCN and 3-MC decreased total serum T₄ concentration by 48 and 18%, respectively. In contrast, PCB treatment markedly reduced total T₄ (90%). Comparable effects were seen in Gunn rats, with decreases in total T₄ of 38, 19, and 91% with PCN, 3-MC, and PCB treatments, respectively. Free serum T₄ concentration in control Gunn rats was 33% higher than in Wistar rats. PCN decreased free serum T₄ in Wistar rats by 47%, whereas PCB treatment decreased free T₄ by 93%. Similar effects were seen in Gunn rats for PCN and PCB treatments, with decreases in free T₄ of 42 and 89%, respectively (Fig. 2, bottom panel).

Total serum T₃ concentration in control Gunn rats was not different from Wistar rats (Fig. 3, top panel). Treatment with PCN and 3-MC had no significant effect on total serum T₃ in either Wistar or Gunn rats. However, PCB treatment decreased total T₃ by 27 and 46% in Wistar and Gunn rats, respectively. Free serum T₃ concentration in control Gunn rats was not different from Wistar rats. Free T₃ concentration in Wistar rats was not affected by PCN and 3-MC treatments, whereas PCB treatment decreased free T₃ (51%). In contrast, free T₃ concentrations in Gunn rats were decreased by 3-MC and PCN treatments by 29 and 61%, respectively (Fig. 3, bottom panel).

Effect of microsomal enzyme inducers on serum TSH

Serum thyroid stimulating hormone (TSH) concentration was similar in both Wistar and Gunn controls (Fig. 4). Treatment with PCN had the most dramatic effect on TSH, with increases of 130 and 277% in Wistar and Gunn rats, respectively. 3-MC treatment had no

significant effect on TSH concentrations in either Wistar or Gunn rats. PCB treatment increased TSH by 72 and 60% in Wistar and Gunn rats, much less dramatically than PCN treatment; however, this increase was only significant in Wistar rats.

Thyroid gland histology of rats treated with microsomal enzyme inducers

Fig. 5 shows micrographs of representative thyroid tissue from control, PCN (800 ppm), 3-MC (200 ppm), and PCB (100 ppm)-treated Wistar and Gunn rats. Thyroid tissue from control Wistar contained cuboidal-shaped thyroid follicular cells, medium-to-large round-shaped colloids, and a few PCNA-labeled nuclei. Thyroid tissue from control Gunn rats also contained cuboidal-shaped follicular cells and few PCNA-labeled nuclei, but colloids were smaller and more irregularly shaped. PCN-treated thyroid tissue from Wistar and Gunn rats similarly contained cuboidal-shaped follicular cells, small-to-medium irregularly shaped colloids, but numerous PCNA-labeled nuclei. Thyroid tissue from both Wistar and Gunn rat strains treated with 3-MC contained cuboidal follicular cells, medium-to-large round colloids, and some PCNA-labeled nuclei, whereas PCB-treated rats also contained cuboidal thyroid follicular small-to-large round and irregularly shaped colloids, and some PCNA-labeled nuclei.

Effect of microsomal enzyme inducers on thyroid weight and follicular cell proliferation

In Wistar and Gunn rats treated with MEI, only PCN treatment increased thyroid weight, by 11 and 20%, respectively, compared to non-treated controls of the same strain (Fig. 6, top panel). Similar to thyroid weight, thyroid follicular cell proliferation, expressed as a labeling index, was also increased only in PCN-treated animals. Thyroid follicular cell proliferation was increased by PCN treatment in both Wistar and Gunn rats by 53 and 43%, respectively, as compared to controls (Fig. 6, bottom panel). Neither thyroid weight nor proliferative activity was appreciably affected by 3-MC or PCB treatment in Wistar and Gunn rats.

Discussion

Glucuronidation is an important metabolic pathway for detoxication, increasing water solubility of substrates, and facilitating their biliary excretion. Hepatic T₄ glucuronidation is impaired in Gunn rats, which have a defect in all Ugt1a enzymes (Beetstra et al., 1991; Visser et al., 1993; Kato et al., 2004, 2007, 2010). Increased T₄ glucuronidation after treatment with MEI results in a decrease in serum T₄ and an increase in TSH (McClain, 1992), which is important in that prolonged TSH increases can lead to the development of thyroid tumors. The present study questions the importance of T₄ glucuronidation in this scenario, using Ugt1a-deficient Gunn rats and the background matched Wistar rats. Glucuronidation of T₄ is quantifiable in both Wistar and Gunn rats, although it is much less in Gunn rats (Fig. 1). T₄ glucuronidation is increased in Wistar rats after MEI treatment but was not increased in Gunn rats. These data confirm that T₄ glucuronidation is performed primarily by Ugt1a enzymes (Beetstra et al., 1991; Visser et al., 1993; Vansell and Klaassen, 2002a,b; Visser et al., 1993; Kato et al., 2004, 2008).

The present study clearly demonstrates that the decrease in serum T₄ produced by MEI treatment is not dependent on Ugt1a enzymes and T₄ glucuronidation. Treatment with PCN, 3-MC, or PCB decreased total serum T₄ in both Wistar and Gunn rats, with the most dramatic decrease after PCB treatment (Fig. 2). Decreases in free serum T₄ were also observed, but were only present in rats treated with PCN or PCB. In a recent study, Kato et al. (2005) found that decreased total T₄ after PB induction in Gunn rats was also not dependent on UGT glucuronidation of T₄. Therefore, it can be concluded that events other than T₄ glucuronidation must be involved in decreasing the T₄ levels observed in Gunn rats.

In contrast to T₄ glucuronidation, T₃ glucuronidation was comparable in Wistar and Gunn strains (Kato et al., 2004, 2005, 2007). Glucuronidation of T₃ is performed primarily by Ugt2b enzymes (Beetstra et al., 1991; Visser et al., 1993; Richardson and Klaassen, 2010). In Ugt1a-deficient Gunn rats, Ugt2b enzyme might be increased as a compensatory mechanism (Wang et al., 2009). Ugt2b enzymes are thought to glucuronidate T₃, and Ugt2b2-deficient Fischer 344 rats and SD rats were studied for T₃ glucuronidation (Richardson and Klaassen, 2010). The results showed that PCN treatment increased T₃ glucuronidation in both SD and Ugt2b2-deficient F344 rats, and increased serum TSH and thyroid follicular cell proliferation, suggesting that T₃ is also glucuronidated by other PCN-inducible Ugt2b enzymes in addition to Ugt2b2 (Richardson and Klaassen, 2010). Generally speaking, T₃ is more hormonally active than T₄, and is more important in maintaining thyroid homeostasis.

In the present study, TSH was increased substantially with PCN treatment in both Wistar and Gunn rats, and increased with PCB treatment in Wistar rats. From the dramatic increase in TSH, but little glucuronidation of T₄ in Gunn rats, it can be concluded that T₄ glucuronidation does not play a major role in increasing TSH and Ugt1a enzymes are not responsible for the increase in TSH.

Studies have investigated the effects of MEI on thyroid follicular cell proliferation in rats. Hood et al. (1999b) observed large increases in thyroid follicular cell proliferation that resulted from small increases in serum TSH. For example, treatment of rats with PB or PCN increased both serum TSH and cell proliferation, while treatment with 3-MC or PCB did not increase TSH and did not consistently increase proliferation (Hood et al., 1999). Compared to other parameters, such as increased thyroid gland weight, cellular hypertrophy, or reductions in colloid space, follicular cell proliferation is the most sensitive indicator of TSH stimulation (Klaassen and Hood, 2001). Increased TSH concentrations (Fig. 4) results in changes in thyroid morphology (Fig. 5), increased thyroid gland weight, and follicular cell proliferation (as labeling index), only after PCN treatment (Fig. 6). As such, implications of thyroid tumor promotion caused by MEI must involve both changes in TSH as well as morphological changes (Vansell et al., 2004).

Several mechanisms including deiodination, biliary excretion, or uptake of thyroid hormones could contribute to the decrease in serum T₄ and the increase in TSH observed after treatment with MEI. Deiodinases produce the majority of T₃ from T₄. An exhaustive investigation of deiodinases in both hepatic and extrahepatic rat tissues surprisingly showed that treatment with PCN, 3-MC, PCB, or PB decreased, rather than increased, deiodinase activity (Hood and Klaassen, 2000b; Kato et al., 2005). Another possible mechanism underlying increased TSH levels after MEI treatment is enhanced biliary excretion of thyroid hormone. Treatment with PCN, 3-MC, or PCB reduced total serum T₄, and increased the overall amount and rate of excretion of T₄ into bile (Vansell and Klaassen, 2001). However, PCN had the least effect on T₄ glucuronidation and biliary excretion compared to the other MEI, but had the largest effect on serum TSH. Vansell and Klaassen (2002a) also showed that PCN increased the excretion of T₃-glucuronide into bile, whereas total serum T₃ was decreased slightly, although T₃ concentration was maintained when compared to serum T₄. Thus, biliary excretion of T₃-glucuronide, not T₄-glucuronide may contribute to increases in TSH observed after inducer treatment.

Transport of T₄ and T₃ in the liver may be critical in maintenance of TH homeostasis (Friesema et al., 2005; Lecureux et al., 2009). Organic anion transporting polypeptides (Oatp1a1, 1a4, and 2b1) (Abe et al., 1999; Friesema et al., 1999; Cattori et al., 2000) could play a role in hepatic uptake of thyroid hormones for their subsequent metabolism. It should be noted that the expressions of hepatic Oatp1a1 and Oatp1a4 are lower in Ugt1a-deficient

Gunn rats (Higuchi et al., 2004). The role of transporters in thyroid hormone homeostasis in Gunn rats needs further investigation.

In summary, it can be concluded from the present data that T₄ glucuronidation is performed primarily by Ugt1a enzymes, due to the very limited glucuronidation in Ugt1a-deficient Gunn rats. Therefore, the glucuronidation of T₄ after MEI treatment in Gunn rats cannot account for the decrease in serum T₄ and subsequent increase in TSH. Thus, none of these parameters are dependent on Ugt1a enzymes, consistent with the literature (Kato et al., 2004, 2005, 2007). Increased T₃ glucuronidation, rather than T₄ glucuronidation, was better associated with increased TSH, suggesting that induction of T₃ glucuronidation and/or possibly increased biliary excretion of glucuronides, as well as thyroid hormone uptake by hepatic transporters, may play a role in regulation of TSH secretion. Additionally, increased TSH concentration after PCN treatment is associated with changes in thyroid morphology and gland weight, as well as follicular cell proliferation.

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Abbreviations

MEI	microsomal enzyme inducer
T₄	thyroxine
T₃	triiodothyronine
TSH	thyroid stimulating hormone
PCN	pregnenolone-16 α -carbonitrile
3-MC	3-methylcholanthrene
PCB	Aroclor 1254
Ugt	UDP-glucuronosyltransferases
UDP-GA	uridine diphosphoglucuronic acid
PB	Phenobarbital

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Fig. 1. Effect of PCN, 3-MC, and PCB on hepatic glucuronidation of thyroxine (T_4 ; top panel) and triiodothyronine (T_3 ; bottom panel) in male Wistar and Gunn rats. Values are mean $\pm$ SE, $n=5-6$. Statistical significance was accepted at $p \leq 0.05$. Symbols indicate significance from control (*; within a strain) or within a treatment group (#; between strains).

Fig. 2. Effect of PCN, 3-MC, and PCB on serum concentrations of total (top panel) and free (bottom panel) thyroxine (T_4) in male Wistar and Gunn rats. Values are mean $\pm$ SE, $n=5-6$. Statistical significance was accepted at $p \leq 0.05$. Symbols indicate significance from control (*; within a strain) or within a treatment group (#; between strains).

Fig. 3. Effect of PCN, 3-MC, and PCB on serum concentrations of total (top panel) and free (bottom panel) triiodothyronine (T₃) in male Wistar and Gunn rats. Values are mean $\pm$ SE, $n=5-6$. Statistical significance was accepted at $p \leq 0.05$. Symbols indicate significance from control (*; within a strain) or within a treatment group (#; between strains).

Fig. 4. Effect of PCN, 3-MC, and PCB on serum concentrations of thyroid stimulating hormone (TSH) in male Wistar and Gunn rats. Values are mean $\pm$ SE, $n=5-6$. Statistical significance was accepted at $p \leq 0.05$. Symbols indicate significance from control (*; within a strain) or within a treatment group (#; between strains).

Fig. 5. Thyroid gland morphology. Representative photomicrographs of thyroid glands from Wistar and Gunn control, PCN, 3-MC, and PCB, respectively. Labeled thyroid follicular cell nuclei are stained brown/black for proliferating cell nuclear antigen. Magnification, $\times 20$.

Fig. 6. Effect of PCN, 3-MC and PCB on thyroid weight (top panel) and thyroid follicular cell proliferation, expressed as labeling index (bottom panel). The thyroid-to-body weight ratio is expressed as g thyroid/kg body weight. Values are mean $\pm$ SE, $n=5-6$. Statistical significance was accepted at $p \leq 0.05$. Symbols indicate significance from control (*; within a strain).