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Promotion of Thyroid Tumors in Rats by Pregnenolone-16α-Carbonitrile (PCN) and Polychlorinated Biphenyl (PCB)

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Pregnenolone-16α-carbonitrile (PCN) and Aroclor 1254 (PCB) both reduce serum thyroid hormone levels in rats, but only PCN consistently produces an increase in serum thyrotropin (TSH). PCN-mediated increases in TSH result in increased thyroid follicular cell proliferation and hyperplasia, which may represent early events on a morphological continuum leading to neoplasia. The purpose of this study was to assess whether PCN, a compound that increases serum TSH, and PCB, which does not increase TSH, promote thyroid tumors in a two-stage carcinogenesis model. Male SD rats were administered the thyroid tumor initiator diisopropanolnitrosamine (2.5 g/kg, sc), and after seven days were fed control diet, diet containing 1000 ppm PCN, or diet containing 100 ppm PCB for 19 weeks. Body weights were unaffected by PCN treatment, but were reduced 21% after 19 weeks of PCB treatment compared to control. PCN treatment significantly reduced serum T_4 through week 3 before returning to control concentrations, whereas T_4 levels following PCB treatment fell below detection limits by week 3 and remained drastically reduced through week 19. TSH concentrations in PCN-treated rats increased threefold at week 2, then declined to near control values at week 19. After one week of PCB treatment, TSH concentrations reached nearly twice that of controls, and were sustained until week 6. The incidence of thyroid follicular cell proliferative lesions, including cystic and follicular hyperplasia, cystic and follicular adenoma, and follicular carcinoma, was significantly increased following PCN treatment, but not following PCB treatment. PCB treatment caused an increase in thyroid carcinomas (4 of 22 rats) not associated with the proliferative-type lesions produced by PCN, despite an increase in TSH serum concentrations. In conclusion, PCN appears to promote thyroid tumors in a manner consistent with known effects of excessive TSH stimulation. However, thyroid carcinomas stemming from PCB treatment indicate that separate mechanisms exist for the production of thyroid cancer in rodents by chemicals classically considered microsomal enzyme inducers.

Key Words: **thyroid; pregnenolone-16α-carbonitrile (PCN); polychlorinated biphenyl (PCB); thyrotropin (TSH); tumor promotion; glucuronidation; rat.**

Few chemicals are known to be directly genotoxic to the thyroid (Hill *et al*., 1989). Nonetheless, neoplastic changes in thyroid gland structure have been produced in rats by a number of stimuli, such as iodine-deficiency (Axelrod and Leblond, 1955), radiation (Doniach, 1953), and treatment with a variety of goitrogenic chemicals, including propylthiouracil (Kitahori *et al*., 1984), perchlorate (Hiasa *et al*., 1987), aminotriazole (Hiasa *et al*., 1982b), and methimizole (Jemec, 1980). Several mechanisms are thought to exist by which these treatments disrupt serum thyroid hormone homeostasis, among them inhibition of thyroid hormone synthesis (Capen, 1997, 2000). However, it is well accepted that subsequent stimulation of thyroid growth occurs secondary to the increased secretion of thyroid stimulating hormone (TSH), the primary regulator of thyroid function by the pituitary (Furth, 1969). Evidence that TSH mediates pathological changes leading to adenoma include (1) the formation of thyroid tumors after transplantation of TSH-secreting pituitary tumors (Dent *et al*., 1956; Sinha *et al*., 1965); (2) recovery of normal thyroid histology upon treatment with exogenous thyroid hormone (Jemec, 1980; McClain *et al*., 1988); (3) prevention of thyroid tumor formation in hypophysectomized rats (Jemec, 1980; Nadler *et al*., 1970); (4) development of pituitary lesions, as well as thyroid lesions following treatment with goitrogens (Axelrod and Leblond, 1955). The morphological changes that occur in response to excessive TSH stimulation represent a continuum from diffuse hyperplasia to nodular hyperplasia, to eventual neoplasia, and are similar irrespective of the stimulus responsible for elevations in TSH secretion (reviewed in Hill *et al*., 1989).

Alteration of the enzymes involved in thyroid hormone metabolism has been described as a mechanism for reducing the concentrations of available thyroid hormone in the circulation (Curran and DeGroot, 1991). Some chemicals that increase the activity of hepatic microsomal enzymes are believed to alter thyroid homeostasis by increasing the glucuronidation and biliary excretion of thyroid hormones (McClain *et al*., 1989; Semler *et al*., 1989). This causes a reduction in serum thyroid hormones, which in turn leads to reduced negative feedback at the pituitary, resulting in elevated TSH secretion. The microsomal enzyme inducer phenobarbital has been shown to increase thyroid gland weight, follicular cell hypertrophy, and hyperplasia (Japundzic, 1969). It has also been found to promote thyroid follicular tumors in a rat two-stage carcinogenesis model (Hiasa *et al*., 1982a;

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McClain *et al*., 1988). The promotion of thyroid tumors by phenobarbital was prevented when rats were supplemented with exogenous T_4. Phenobarbital-induced hyperplasia was also prevented by exogenous T_4, and prevented by hypophysectomy as well (Japundzic, 1969). It was subsequently determined that the effect of PB on thyroid function was primarily due to the increased hepatic clearance of T_4, likely to be secondary to induction of glucuronidation.

The nature of this mechanism has been further investigated by examining the effects of other microsomal enzyme inducers on TSH secretion, thyroid growth, and thyroid follicular cell proliferation in rats (Hood *et al*., 1999a,b). While there is a direct relationship between the changes in TSH and changes in thyroid cell proliferation, the effects on T_4 glucuronidation are not consistent with either parameter (Hood *et al*., 1999b; Hood and Klaassen, 2000a). Certain inducers, namely phenobarbital and pregnenolone-16α-carbonitrile (PCN), increase T_4 glucuronidation as well as increase serum TSH and thyroid cell proliferation. Two other inducers, 3-methylcholanthrene (3MC) and the polychlorinated biphenyl mixture Aroclor 1254 (PCB), increase T_4 glucuronidation as well, but do not remarkably increase serum TSH or thyroid cell proliferation in rats. Recently, it was reported that the effects of these four inducers on T_3 glucuronidation are consistent with the effects on TSH (Hood and Klaassen, 2000a; Vansell and Klaassen, 2002). Based on these findings, it was hypothesized that the ability to increase T_3 glucuronidation—rather than T_4 glucuronidation—might be a better predictor of the effects of these inducers on TSH secretion.

Presently, it is unknown whether any of the aforementioned inducers, besides phenobarbital, are promoters of thyroid tumors in rats. Therefore, the purpose of this study was to determine whether chemicals that induce the glucuronidation of T_3 produce elevations in serum TSH levels and promote thyroid follicular tumors. A 20-week initiation-promotion model of carcinogenesis similar to that utilized by Hiasa *et al*. (1987) and McClain *et al*. (1988) was used. It was expected that PCN, by increasing T_3 glucuronidation, would increase serum TSH, resulting in thyroid tumors, whereas PCB, which has no effect on T_3 glucuronidation and on serum TSH, would not.

MATERIALS AND METHODS

Chemicals and reagents. 16-Dehydropregnenolone was obtained from Steraloids, Inc. (Newport, RI) and Aroclor 1254 (PCB) was donated by Dr. Larry Hansen (University of Illinois at Urbana-Champaign). Pregnenolone-16α-carbonitrile (PCN) was synthesized from 16-dehydropregnenolone as previously described (Sonderfan and Parkinson, 1988). Diisopropanolnitrosamine (DIPN), also known as *N*-bis-(2-hydroxypropyl)-nitrosamine (DHPN) was obtained from Ash Stevens, Inc. (Detroit, MI). Radioimmunoassay kits for total T_3 and T_4 were obtained from Diagnostic Products Corp. (Los Angeles, CA), and kits for rat TSH was obtained from Amersham Life Science, Inc. (Arlington Heights, IL). All other reagents were obtained from Fisher Scientific (Pittsburgh, PA).

Animals and treatments. Male, Sprague-Dawley rats (Sasco, Wilmington, MA) weighing 150–200 g were divided into three groups of 24 and one group of eight based on matched body weights. Animals were housed individually in wire-bottom cages with *ad libitum* food (Purina 5002 chow containing 2.5% corn oil) and water. A 20-week initiation-promotion model was utilized as described by Hiasa *et al*. (1987). Following a one-week acclimation period, rats were given a single injection of DIPN (2.5 g/kg body weight sc) or saline, and allowed a one-week recovery period. Saline-injected rats were then placed on control diet ($n = 8$), and initiated rats were placed on control diet ($n = 24$), diet containing 1000 ppm PCN ($n = 24$), or diet containing 100 ppm PCB ppm ($n = 24$) for 19 weeks. Diets were synthesized by DYETS, Inc. (Bethlehem, PA) from Purina 5002 chow and supplemented with 2.5% corn oil. Rats were fed and observed daily and body weights were recorded weekly through day 28, then at least biweekly thereafter. Overnight food consumption was recorded periodically throughout the study. At the end of 19 weeks, rats were decapitated and liver, thyroid, and pituitary were removed, weighed, and both thyroid lobes were fixed in 10% neutral formalin overnight for histological processing. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee.

Histopathology. Following 24 h fixation in 10% neutral formalin, thyroids were processed using routine histological techniques. Paraffin-embedded thyroids (five thyroids per block in fixed orientation) were sectioned serially at 5 microns and stained with hemotoxylin and eosin by HistoTechNology, Inc. (Cleona, PA), with every third section excluded. For the eight thyroids from saline-injected control animals, all sections were examined for the presentation of pathology. Five serial sections were selected at random and blinded for analysis of the thyroids from the remaining groups (initiated control, PCN, and PCB). This is because each block contained five thyroids of varying size in each block, and therefore a given serial section did not represent the same depth of penetration for each thyroid. Of these five sections, the section with the most severe histopathology was used in the scoring for each rat.

Determination of serum T_3, T_4, and TSH. Blood was sampled from the retro-orbital sinus on days −1, 7, 14, 21, 42, 91 relative to the start of diet treatment, and trunk blood was collected at necropsy on day 133. After clotting at 4°C, blood samples were centrifuged for 5 min to collect serum, which was stored at −80°C until further analysis. The concentrations of total (free and protein-bound) T_3 and T_4, as well as TSH, were determined by radioimmunoassay. Limits of detection for these kits were 7 ng/dl, 0.25 μg/dl, and 0.50 ng/ml, respectively. When adequate quantities of serum could not be obtained from all rats, samples were distributed to ensure that at least 10 samples/group/timepoint were analyzed for each parameter.

Data analysis. Body and organ weights, food consumption, and serum hormone concentrations are expressed as the mean ± SE. Means were compared by one-way ANOVA followed by Duncan's multiple range test. Statistical significance is reported for the $p < 0.05$ level. For histopathology incidence, lesions were scored as either present or absent, and results are expressed as the number of thyroids with each lesion present out of the total number of thyroids examined for each group. Incidences were compared by Fisher's exact p two-tailed test, and significance is reported at the $p < 0.05$ level.

RESULTS

Results are presented for initiated groups only (control, PCN, PCB), though an additional eight saline injected control animals were included in the study to distinguish any effect of DIPN alone. All timepoints are relative to the initiation of diet treatment. Two PCB-treated rats died prematurely in week 9. Data obtained from these animals up to the time of death are included in calculations of means and statistical evaluations; however, organ weights and histopathology were not determined for these animals.

Mean body weights of initiated rats fed either control diet or diet containing PCN or PCB for 19 weeks (133 days) are given in Figure 1. At day −8, the day prior to initiation with DIPN, mean

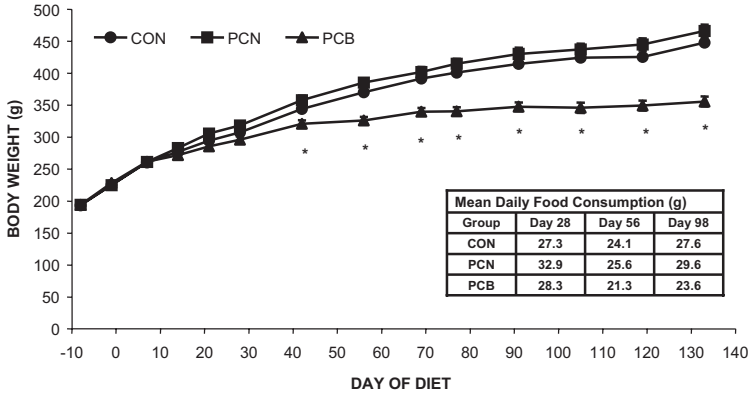

FIG. 1. Effect of microsomal enzyme inducers pregnenolone-16α-carbonitrile (PCN) and Aroclor 1254 (PCB) on body weight over 19 weeks of treatment. Table inset: effect of PCN and PCB treatment of daily food intake. Values are the mean ± SE. *Significantly different from initiated controls (CON; $p < 0.05$).

body weights for all groups were not different (194 g), and there were no differences in mean body weight among the three initiated groups from day −1 to day 27 of treatment. Whereas there were no differences between control and PCN-treated rat body weights throughout the course of the study, a reduction in mean body weight gain in PCB rats was apparent by day 42 and was sustained for the duration of the study. On day 42, the mean body weight of PCB rats was 7% less than that of controls, and was 21% less than mean control group weight by day 133.

The mean daily food consumption among these groups, however, was not different. Food consumption on days 28, 56, and 98 is also shown in Figure 1. There were no differences between the gram quantity of food consumed in 24 h, and consumption was even slightly greater for PCB-treated animals when considered on a body weight basis.

Serum thyroid hormone concentrations were also monitored throughout the course of the experiment, and are shown in Figure 2. Within seven days of diet treatment, serum total T_4 concentrations, in the top panel, were significantly reduced after PCN and PCB treatments. These reductions were sustained in both PCN and PCB-treated rats through day 21, decreasing to only 40% of controls in PCN-treated rats. These values returned to control levels between days 21 and 42 in PCN-treated rats, but were significantly lower than control rat T_4 concentrations again at day 133. Serum T_4 reductions in PCB-treated rats were much more dramatic. On day 21 of treatment, T_4 concentrations were below the limits of detection, and remained drastically reduced through day 133, when T_4 levels were only 10% that of control animals.

Serum T_3 concentrations, in general, were more variable and less clearly affected by either treatment (Fig. 2, middle panel). Prior to the start of treatment (day −1), serum total T_3 concentrations in the PCN group were significantly lower than controls, though all groups were untreated at this point. During treatment, T_3 concentrations were significantly reduced only in PCB-treated animals on days 14 and 133.

Serum TSH concentrations, like those of T_4, were increased after seven days of PCN and PCB dietary administration (Fig. 3, bottom panel). PCN treatment produced the largest elevations in TSH concentrations, peaking at three times control on day 14. TSH concentrations then returned to control levels on day 42, but were again higher than control on days 92 (significantly) and 133. PCB treatment induced a more sustained elevation in serum TSH between days 7 and 133; however, the increase was not as dramatic as that initially produced by PCN treatment. The average increase in TSH serum concentrations in PCB-treated animals between days 7 and 133 was 69%.

Figure 3 depicts the ratio of thyroid, pituitary, and liver weight to body weight at the end of 19 weeks of diet. Both PCN (by 71%) and PCB (by 236%) exposure increased the mean thyroid to body weight ratio (top panel). It should be noted that two thyroids from PCB-treated rats (196 mg and 467 mg), weighed well above the average for remaining PCB-treated rats (29.7 mg), and skewed the mean for that group. Median absolute thyroid weights were: control, 23 mg; PCN, 41 mg; PCB, 26 mg. No significant changes in pituitary weight were evident following PCN or PCB treatment (Fig. 3, middle panel). Both PCN and PCB significantly increased the liver to body weight ratios above that of controls: PCN by 36%, and PCB by 94%.

Thyroids were evaluated histologically for the presence of follicular cell pathologies. Thyroids from eight saline-injected rats maintained on control diet were first examined to obtain a reference for normal histological appearance of the gland. Figure 4, panel A depicts the normal thyroid architecture from a saline-injected control animal. Fairly uniform follicle size and even staining are apparent. Follicles are lined with cuboidal to low-columnar epithelium. Generally, there were no dramatic differences between saline-injected control thyroids and initiated control thyroids. Follicles consisted primarily of cuboidal to low columnar epithelium with evidence of mild diffuse hyperplasia. The epithelium of some larger follicles was flattened cuboidal to squamous. Large follicles were located

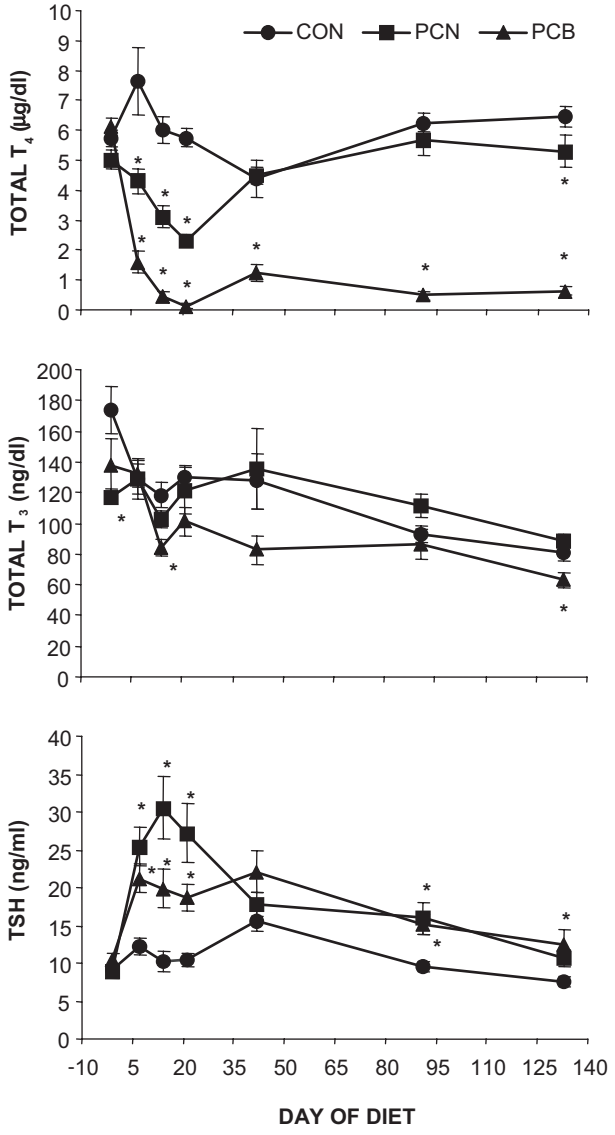

FIG. 2. Effect of pregnenolone-16α-carbonitrile (PCN) and Aroclor 1254 (PCB) on serum concentrations of total T_4 (top panel) and T_3 (middle panel), and TSH (bottom panel) over 19 weeks of treatment. The first timepoint is one day prior to the start of diet treatment (day −1). Values are mean ± SE. *Significantly different from initiated controls (CON; $p < 0.05$).

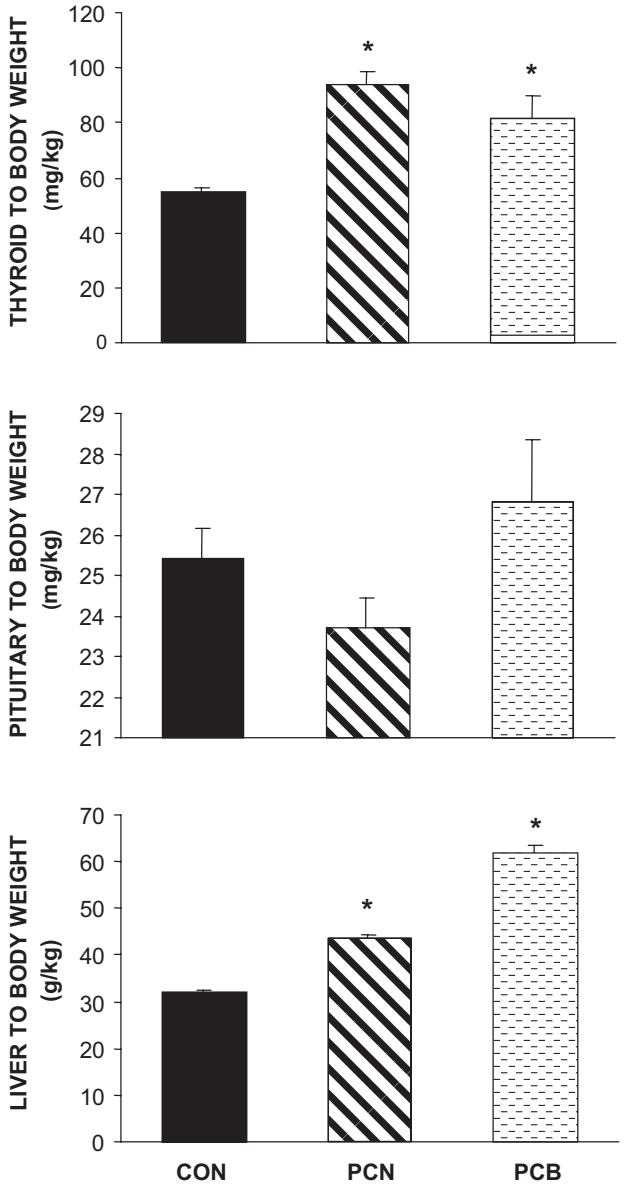

FIG. 3. Effect of 19 weeks of treatment with pregnenolone-16α-carbonitrile (PCN) or Aroclor 1254 (PCB) on thyroid weight (top panel), pituitary weight (middle panel), and liver weight (bottom panel). Thyroid and pituitary weights are expressed as mg/kg body weight; liver weight is expressed as g/kg body weight. Values are mean ± SE. *Significantly different from initiated controls (CON; $p < 0.05$).

only on the outer edge of some glands, while other thyroids contained large follicles within the center of the gland. There was also evidence of vacuolization of the colloid and cytoplasm in a number of thyroid glands.

Panels B–G of Figure 4 are photomicrographs representing the proliferative lesions (consistent with excessive thyroid stimulation) identified in this study. These foci were categorized as follows: cystic hyperplasia, focal hyperplasia (localized hyperplasia occurring in nodules of >1 follicle that was well-demarcated from surrounding tissue), cystic adenoma, follicular adenoma (adenoma of follicular to solid pattern), and follicular carcinoma (localized carcinoma with follicular or mixed pattern).

Panel B contains a lesion of cystic hyperplasia. The colloid space is dilated with papillary projections into the cystic space; there is slight compression of the surrounding tissue due to the expanded volume of the colloid space. Panel C shows a nodule of hyperplastic follicles. There is distinct demarcation from the surrounding tissue without compression; colloid-filled follicles are irregularly shaped with cuboidal to columnar epithelium and slight papillary infolding. A second type of focal hyperplasia that was noted in these thyroids contained smaller, more hyperchromatic follicles with decreased colloid and darker nuclei than the

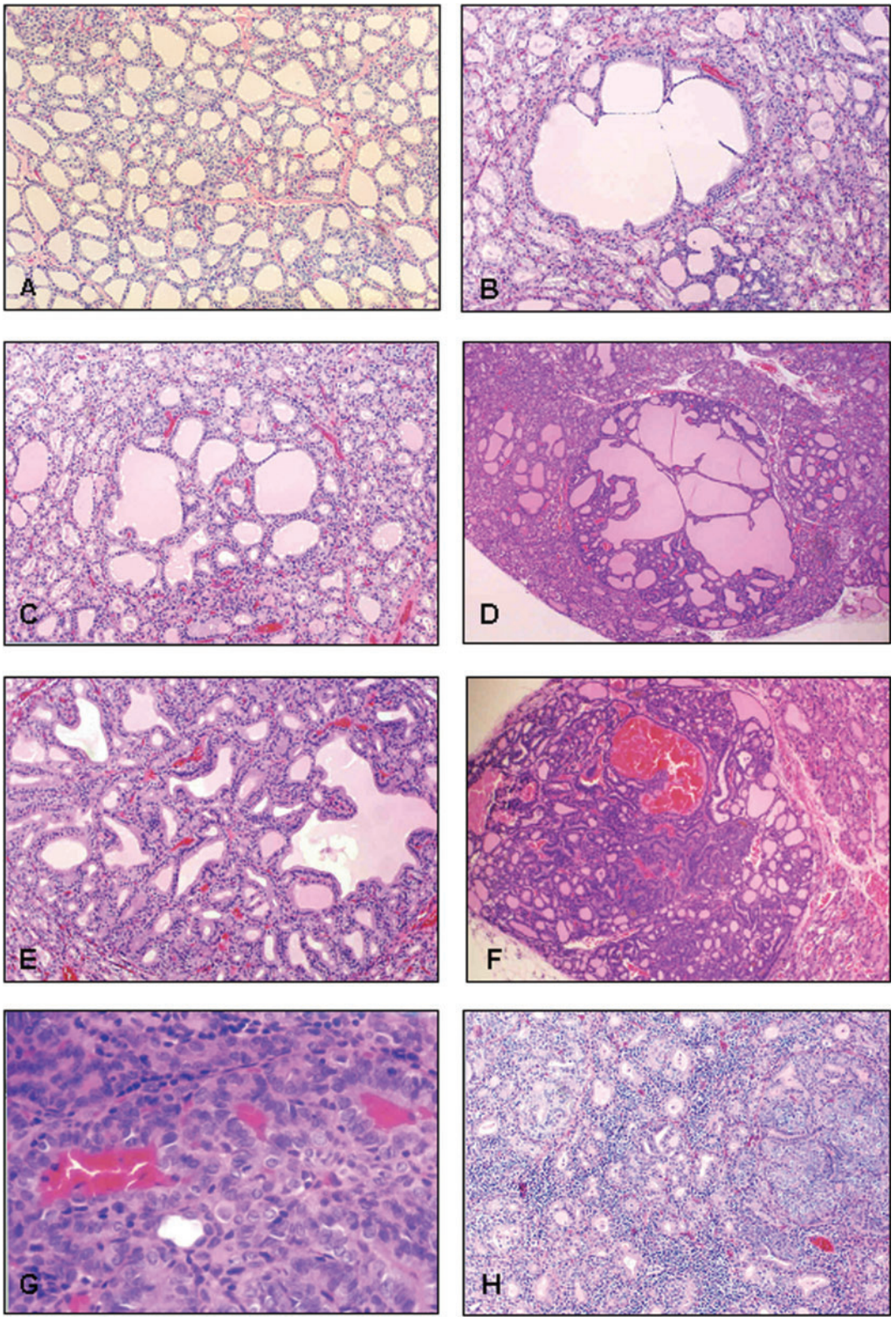

FIG. 4. Representative photomicrographs of each type of lesion identified in rat thyroid at the end of 19 weeks of treatment with pregnenolone-16α-carbonitrile (PCN) or Aroclor 1254 (PCB). (A) General appearance of a saline-injected control thyroid, 100X; (B) cystic hyperplasia in a PCN thyroid, 100X; (C) focal hyperplasia in a PCN thyroid, 100X; (D) cystic adenoma in a PCN thyroid, 40X; (E) follicular adenoma in an initiated control thyroid, 100X; (F) follicular carcinoma in a PCN thyroid, 100X; (G) follicular carcinoma in a PCN thyroid, 400X; (H) complete carcinoma in a PCB thyroid, 100X.

TABLE 1
Group Incidence and Frequency of Altered Thyroid Foci after 19 Weeks of Dietary Treatment with Pregnenolone-16α-carbonitrile (PCN) or Aroclor 1254 (PCB)

Group	Number of rats	Cystic hyperplasia	Focal hyperplasia	Cystic adenoma	Follicular adenoma	Follicular carcinoma	Complete carcinoma
CON	24	3 (0.1)	6 (0.3)	0 (0)	5 (0.3)	1 (0)	0
PCN	24	17* (3.6)	24* (9.3)	15* (1.1)	21* (3.1)	10* (0.5)	0
PCB	22	5 (0.5)	12 (1.7)	2 (0.1)	9 (0.6)	0 (0)	4*

Note. By definition, the number of complete carcinomas per thyroid can only equal 1. ( ): Average number of lesions observed per thyroid section.
*$p < 0.05$, significantly different from controls.

focus shown in Panel C. Part of a cystic adenoma is shown in Panel D. It is well circumscribed with a thin capsule and formation of an irregular, complex network of projections into the cystic lumen. The follicular adenoma in panel E is characterized by an irregular but single morphological pattern of hyperchromatic follicles with nuclear crowding, partial encapsulation, and compression of surrounding tissue. Other adenomas included in this classification exhibited a more solid pattern of densely packed cells with a high nuclear to cytoplasm ratio, stained more basophilic, with some atypical nuclei and mitotic figures present. Panels F and G show two follicular carcinomas at two different magnifications. At lower power, there is evidence of a heterogeneous growth pattern, with prominent vascularity and cellular pleomorphism. At higher magnification, there is evidence of a well-differentiated neoplasm, atypical nuclei, vascularization, and mitotic figures.

A sixth classification, termed complete carcinoma, was established to describe the unique histopathology detected in some PCB-treated rats. These lesions differed dramatically from that of the localized follicular carcinomas also observed in this study. A small portion of a complete thyroid carcinoma is shown in Panel H. Extreme lymphocytic invasion and mixed cell types and patterns are visible, with no normal recognizable thyroid architecture remaining. The entire gland consisted of such architecture, extending beyond the normal thyroid capsule. The histological appearance and severity of this lesion was consistent among thyroids in which it was detected, and no pre-neoplastic lesions indicative of earlier stages of progression to such complete carcinomas were observed.

The incidence of each lesion in thyroids from (initiated) control, PCN-, and PCB-treated rats is presented in Table 1, including the mean number of lesions observed per thyroid section in parenthesis. Cystic hyperplasia, focal hyperplasia, follicular adenoma, and follicular carcinoma were present in thyroids from control rats. The incidence of all proliferative-type lesions was increased above control incidence following PCN treatment, including 100% incidence of focal hyperplasia, as well as the mean number of proliferative foci per section. PCB treatment produced only a slight, but not statistically significant, increase in the incidence and mean frequency of these lesions. However, 4 of 22 PCB thyroids were entirely transformed such that the origin of neither the tissue nor the lesion could be readily identified microscopically, termed complete carcinomas. This was the only lesion that was observed at a significantly increased incidence in thyroids from PCB-treated animals, and was completely absent from thyroids of PCN-treated animals.

DISCUSSION

TSH is the primary regulator of thyroid function as well as the main growth factor for the thyroid (Thomas and Williams, 1991). In rats, excessive TSH stimulation of the thyroid, via maintenance on a low-iodine diet, transplantation of TSH-secreting pituitary tumors, or by exposure to chemical stimuli that increase TSH, such as perchlorate or propylthiouracil, produces proliferative changes in thyroid follicular cells that represent a morphological continuum from hyperplasia to adenoma to carcinoma (Axelrod and Leblond, 1955; Capen, 1997; Dent *et al*., 1956; Sinha *et al*., 1965). Thyroid tumor formation in a rodent two-year bioassay has been demonstrated with several anti-thyroid chemicals that elevate TSH, as well as spironolactone, a microsomal enzyme inducer believed to increase TSH secondary to increasing glucuronidation of thyroid hormone (Lumb *et al*., 1978). The capacity of a second microsomal enzyme inducer, phenobarbital, to promote thyroid tumors has also been demonstrated in an initiation-promotion model (Hiasa *et al*., 1982a; McClain *et al*., 1988). However, the tumor-promoting potential of other classical microsomal enzyme inducers, such as PCN and the PCB mixture Aroclor 1254, has not been evaluated. Treatment of rats with PCN for seven days increased TSH and lead to increased thyroid follicular cell proliferation and hyperplasia (Hood *et al*., 1999a), two events that occur early in the continuum of proliferative changes in thyroid follicular cells. Treatment of rats with the PCB mixture Aroclor 1254 for the same period of time did not elevate serum TSH levels, nor induce thyroid cell proliferative changes. In the present study, the ability of PCN and PCB to promote thyroid tumors in rats has been evaluated utilizing a two-stage initiation-promotion model of carcinogenesis. The nitrosamine DIPN, a genotoxic compound that exhibits a low level of thyroid tumors when given alone but which does not alter the hypothalamic-pituitary-thyroid axis (Hiasa *et al*., 1982b; Mohr *et al*., 1977), was utilized as the initiating agent.

Because PCN increases the glucuronidation of T_3, serum TSH, and cell proliferation, it was hypothesized that in this study, PCN would promote the formation of thyroid follicular tumors in rats. Data collected in this study confirmed that hypothesis. Effects of PCN on liver and thyroid gland weight, serum thyroid hormones, and TSH were consistent with previous observations. Serum TSH concentrations peaked in PCN-treated rats on day 14 of diet, but continued to be greater than those of the control group throughout the course of the study. Effects of PCN on thyroid gland morphology were consistent with those described for other stimuli that mediate increases in TSH. In PCN-treated rats, the incidence of pre-neoplastic nodules was 100% with a mean of approximately 12 hyperplastic foci per section, and the incidence of follicular adenoma was 87.5% with a mean of greater than four adenomas per thyroid section. The incidence of follicular carcinoma (42%) was also significantly increased above that induced by DIPN alone. Taken with previously published data indicating that biliary excretion of T_3 metabolites, particularly T_3-glucuronide, is increased by PCN in rats (Vansell and Klaassen, 2002), these data suggest that PCN acts on the thyroid via a secondary mechanism to promote the transformation of thyroid lesions from hyperplasias to carcinomas.

Effects of the PCB mixture, Aroclor 1254, on thyroid were also evaluated in this study. Because data previously collected by this laboratory indicated this PCB mixture does not increase the glucuronidation of T_3, serum TSH, or thyroid cell proliferation, the expectation was that it would fail to promote thyroid tumors. Whereas the results of this study did not indicate that PCB produces proliferative changes in thyroid follicular cells or tumors that are proliferative in nature, the results also demonstrated that the action of PCB on the thyroid gland is more complicated than presumed. In the present study, PCB increased serum TSH concentrations, albeit not to the degree produced by PCN treatment. In the majority of previously reported rat studies, the PCB mixture utilized, Aroclor 1254, did not increase serum TSH. The concentration selected for the current study was 100 ppm, a dose that has significantly decreased T_4 serum concentrations, but which previously has not elicited increases in TSH. However, higher dietary concentrations of Aroclor 1254 than those used in this study have resulted in elevated serum TSH. At days 14 and 20, 250 ppm resulted in an increase in serum TSH (Barter and Klaassen, 1994), although concentrations of 200 ppm and 300 ppm in two subsequent studies did not increase serum TSH (Hood *et al*., 1999a; Liu *et al*., 1995). It may be the case that accumulation of PCBs over the duration of 19 weeks resulted in a larger effective dose than that for rats fed similar concentrations for shorter lengths of time, particularly considering the decrease in body weight gain observed in PCB-treated rats. This, along with the apparent systemic toxicity produced by PCB (as evidenced by two early mortalities), likely contributed to the drastic reductions of total T_4 serum concentrations, and may have been a greater factor in elevating serum TSH than in previous studies. It is also important to point out, though, that Aroclor 1254 is a mixture of different structural congeners, with varying phenobarbital and TCDD-like chemical properties (Parkinson *et al*., 1983). It is possible that differing contributions of individual PCB congeners or their metabolites could have contributed to the increase in TSH in this study (Kato *et al*., 2000).

Despite the elevation of serum TSH produced by PCB, however, there was not a significant increase in the incidence of proliferative thyroid lesions typically associated with excessive TSH stimulation. The most striking finding was the occurrence of thyroid carcinomas in thyroids from four PCB-treated rats that did not exhibit histomorphology consistent with the thyroid tumors observed in PCN-treated rats, and which did not correlate with increased serum TSH concentrations in individual rats. Thyroids from the 18 remaining PCB-treated rats were fairly similar to those of initiated controls, and did not exhibit the full continuum of proliferative changes induced by excessive TSH secretion. Rather, in the case of PCB, there was evidence that anti-thyroid activity may have blunted the TSH responsiveness of the thyroid. In thyroids from PCB-treated rats that were not considered carcinogenic, there appeared to be an increase in the number of cystic follicles (different than cystic hyperplasia in that the follicle is lined with squamous epithelium, and there is no increase in cell number), indicative of *inactivity*, and increased vacuolization of the colloid and epithelial cytoplasm (data not quantified). Both PCB and polybrominated biphenyl (PBB; Firemaster) mixtures have been shown to produce changes in thyroid histology that may indicate interference with normal thyroid synthesis and secretion, such as increased vacuolization and accumulation of colloid droplets and abnormal lysozymes in follicular cells (Collins *et al*., 1977; Kasza *et al*., 1978). PBBs appear to preferentially accumulate in thyroid, where they have been proposed to bind to thyroid macromolecules and interfere with the organification of iodide (Allen-Rowlands *et al*., 1981). Byrne and colleagues (1987; Sepkovic and Byrne, 1984) demonstrated that disturbances in thyroid hormone synthesis and distribution might occur following long-term administration of PCB and PBB. In addition to an increase in the biological half-life and distribution space for T_3 and T_4, they demonstrated diminished response of serum T_3 and T_4 levels to exogenous TSH injections. These findings were interpreted to be evidence not of thyroid-pituitary axis suppression, but of direct thyroid damage. If PCBs do exert a direct anti-thyroid effect, this could possibly desensitize the thyroid to TSH stimulation, perhaps in a way that would prevent typical proliferative changes.

Certainly, such an anti-thyroid effect would also contribute to the reduction in T_4 serum concentrations. This idea does not appear to agree with the finding that PCB decreases serum T_4 primarily by an extrathyroidal mechanism (Barter and Klaassen, 1992), presumably via increased biliary excretion of T_4 (Vansell and Klaassen, 2001). However, there is evidence that multiple processes are contributing. In studies using the homozygous Gunn rat that possesses a deficiency in T_4 glucuronidation and biliary clearance, T_4 serum concentrations were reduced to the same extent following PCB treatment as in heterozygous rats (Collins and Capen, 1980). Histologically, thyroids from

homozygotes had changes suggestive of impaired hormone secretion, while heterozygote thyroids appeared to be stimulated. Thus, multiple mechanisms must be involved. In the aforementioned study (Byrne *et al*., 1987), a reduction in T_4 production rates was noted, and the authors suggest that this, along with cell membrane damage associated with PCB and PBB intoxication may have expanded pools for T_4 dilution, and therefore contributed to reductions in serum T_4. It has also been suggested that the thyrotoxic effects of PCBs are related to the similarity between the structures of individual PCB congeners and thyroid hormones (McKinney, 1989; Rickenbacher *et al*., 1986). PCB congeners and/or their hydroxylated metabolites interfere with plasma, cytosolic, and nuclear T_4 binding (Brouwer and van den Berg, 1986; McKinney *et al*., 1987; Rickenbacher *et al*., 1986). Such interference could displace T_4 from serum transthyretin (Chauhan *et al*., 2000; Cheek *et al*., 1999; Darnerud *et al*., 1996) and enhance tissue uptake, as would induction of transporter expression. It has also been proposed that PCBs may bind to thyroid hormone receptors in the pituitary to blunt TSH release (Morse and Brouwer, 1994; Rickenbacher *et al*., 1986). Whether these binding properties could also attribute to direct thyroid gland impairment is not known.

The fact that serum T_4 concentrations appear to be completely ablated with virtually no consequence on serum T_3 is particularly interesting, considering nearly 80% of T_3 is derived from deiodination of T_4 (Visser, 1990), and deiodination of T_4 is generally inhibited, rather than enhanced by PCB (Hood and Klaassen, 2000b). This suggests the involvement of mechanisms for the maintenance of serum T_3 concentrations. It has been shown that during thyroid hormone imbalance, *de novo* synthesis and secretion of T_3 by the thyroid may take place in preference to synthesis of T_4 (Delange and Ermans, 1996; Kaplan, 1980). The role of individual PCBs in mediating such a process requires further investigation. However, free T_4 serum concentrations were not determined in the present study, and therefore the physiological impact of such a large reduction in total T_4 serum concentrations cannot be completely assessed.

Previously, it was reported that the effects of microsomal enzyme inducers on the glucuronidation of T_4, and the subsequent reductions in serum T_4 produced by inducer treatment, are inconsistent with the effects on serum TSH (Hood and Klaassen, 2000a). However, increases in T_3 glucuronidation produced by microsomal enzyme inducers do correlate with elevations in serum TSH. It therefore reasons that changes in T_3 glucuronidation, rather than T_4 glucuronidation, may regulate increases in TSH secretion and thyroid cell proliferation. Further, inducers that increase T_3 glucuronidation and serum TSH should then promote thyroid tumors (PCN), while inducers that do not increase T_3 glucuronidation and serum TSH should not promote thyroid tumors. The results of this study partially support this hypothesis, in that PCN increased the number and incidence of thyroid proliferative and neoplastic lesions consistent with excessive TSH stimulation, whereas PCB did not. However, PCB produced thyroid carcinomas in four rats, an occurrence that appears to be unrelated to the elevations in serum TSH also observed. It is not completely clear as to why PCB did not promote thyroid follicular tumors in a manner similar to PCN when both treatments elevated serum TSH, although there is some indication PCB may directly impair thyroid function. When previously studied, this PCB mixture, Aroclor 1254, had no effect on serum TSH, thyroid cell proliferation, or thyroid hyperplasia (Hood *et al*., 1999b). Whether TSH serum concentrations do not reliably indicate thyroid gland stimulation, or whether PCBs may act directly to inhibit the action of TSH on the thyroid, is unknown. Nonetheless, the data herein clearly indicate that (1) PCN mediates a different type of thyroid tumor than PCB and by a different mechanism, and (2) that tumorigenesis in the thyroid is more complicated than can be predicted from serum TSH in the case of chemicals that may exert other thyrotoxic effects. The mechanisms of both PCN- and PCB-induced thyroid neoplasia require further characterization. Evaluation of other inducers of T_3 glucuronidation as potential thyroid tumor promoters is necessary, as well as studies to examine whether supplemental T_3 alone can prevent PCN-induced thyroid tumors, as it has been demonstrated for methimizole (Jemec, 1980). Such evidence would aid in determining the potential for a chemical to elicit thyroid disturbances secondary to excessive TSH stimulation. It is also important to begin to consider other direct actions of microsomal enzyme inducers, in particular individual PCB congeners, that may lead to thyroid tumor formation, and that are independent of TSH stimulation.

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