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Sex Differential and Dose Dependence of Phenobarbital-promoting Activity in *N*-Bis(2-hydroxypropyl)nitrosamine-initiated Thyroid Tumorigenesis in Rats[1]

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ABSTRACT

Studies were made on the dose and sex dependence of thyroid tumor development in rats pretreated with *N*-bis(2-hydroxypropyl)nitrosamine (DHPN) followed by exposure to various doses of phenobarbital (PB). A direct dose-response relationship in induction of thyroid tumors was found in both male and female rats. Upon feeding the DHPN-treated rats with basal diet containing 20, 100, 500, and 2500 ppm of PB, the incidences of follicular adenoma were, respectively, 8, 45, 70, and 66% in male rats and 12, 17, 50, and 58% in female rats. Development of papillary adenomas in male rats was observed only at the higher doses of PB, at incidences of 12 and 20% for doses of 500 and 2500 ppm. Follicular carcinoma was also seen at higher doses of PB, at 16 and 12%, respectively, for the 500- and 2500-ppm groups. Neither follicular nor papillary carcinomas were induced in female rats; only a low incidence of papillary adenoma (4%) was observed with a PB concentration as high as 2500 ppm. A single injection of DHPN resulted in production of approximately 1 tumor/female rat and 2.5 tumors/male rat. DHPN combined with posttreatment with PB at doses up to 500 ppm did not increase tumor yield in female rats, whereas a 3-fold increase was observed in male rats for the 500-ppm-treated groups. When PB was increased to 2500 ppm a marked increase (8-fold) in tumor yield in male rats was observed, in contrast to a <3-fold increase in similarly treated female rats.

INTRODUCTION

The promoting effects of PB[3] on experimental hepatocarcinogenesis have been well documented (9, 13, 16, 20, 22–24) since the original report by Peraino *et al.* (19). However, there are few reports concerning the potential promoting effects of PB on experimental carcinogenesis in other organs. Indeed it has been suggested that the enhancing activity of PB on carcinogenesis may be restricted to the liver (22) while, in contrast, 12-*O*-tetradecanoylphorbol-13-acetate has been reported to exert promoting activity in the liver, lung, and stomach as well as the skin (1–4, 15).

DHPN is a potent carcinogen which induces tumors not only in the thyroid but also in the lung, kidney, and esophagus of rats (14). Whereas no thyroid tumors were observed in rats given weekly injections of a subeffective dose (70 mg/100 g body weight) of DHPN for 12 weeks (18), we have recently shown that multiple injections of this low dose of DHPN when followed by exposure to PB or 3-amino-1,2,4-triazole are effective in inducing thyroid tumors in male rats (7, 8). Furthermore we demonstrated that a single dose of DHPN (280 mg/100 g) when combined with subsequent exposure to PB for at least 12 weeks results in a high incidence of thyroid tumors in the treated rats (6). As part of our ongoing attempts to understand the mechanism of two-stage carcinogenesis in rat thyroid, we decided (*a*) to determine the dose dependency of PB promotion of the development of thyroid tumors initiated by single dose of DHPN and (*b*) to investigate whether the sex of the animal exerts any influence on tumor yield in this experimental model.

MATERIALS AND METHODS

Chemicals and Diet. DHPN and PB were purchased from Nakarai Chemical Co., Kyoto, Japan. PB was added to the basal diet (Oriental MF; Oriental Kobo Co., Osaka, Japan) at various concentrations as specified in the experimental regimen.

Animals. A total of 250 male and 250 female inbred Wistar rats, 6 weeks old (140–170 g), were purchased from Kitayama Labes Animals Co., Kyoto, Japan.

Experimental Design. Chart 1 illustrates the experimental regimen. We selected 240 male and 240 female healthy rats, and each sex was divided into four groups; Groups I and III were further divided into four subgroups, so that each group or subgroup contained 24 rats. Group treatments were as follows: Group I-1, DHPN followed by 2500 ppm PB; Group I-2, DHPN followed by 500 ppm PB; Group I-3, DHPN followed by 100 ppm PB; Group I-4, DHPN followed by 20 ppm PB; Group II, DHPN alone; Group III-1, 2500 ppm PB; Group III-2, 500 ppm PB; Group III-3, 100 ppm PB; Group III-4, 20 ppm PB; Group IV, basal diet only.

PB was administered by admixing into the basal diet. Basal diet and diet containing the drug were given *ad libitum*. In a preliminary experiment we established that female rats required approximately twice the amount of DHPN to induce the same yield of tumors as that for male rats. Thus while DHPN was injected i.p. into male rats at a dose of 210 mg/100 g body weight, this was increased to 420 mg/100 g given to the female rats.

The animals were housed in wire cages in an air-conditioned room at 24°C and weighed weekly. One female rat in Group I-3 died prematurely and was not included into the effective number of rats. At the end of the experiment food was withheld for 16 h, and then the animals were sacrificed under ether anesthesia. After complete autopsy including careful macroscopic examination, the liver, lung, kidneys, and thyroid were weighed and fixed in 10% buffered formalin.

Serial sections were cut through the thyroids of all animals. Sections were routinely stained with hematoxylin and eosin, and specific stains, such as periodic acid-Schiff, van Gieson, and Grimelius, were used in some cases. The number of thyroid tumors was counted in serial sections. The histological classification of thyroid tumors by Napalkov (17) was used in the present experiment.

RESULTS

General Observations. Weight gain was similar for both male and female rats in groups treated with DHPN in combination with

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[3] The abbreviations used are: PB, phenobarbital; DHPN, *N*-bis(2-hydroxypropyl)nitrosamine; TSH, thyroid-stimulating hormone.

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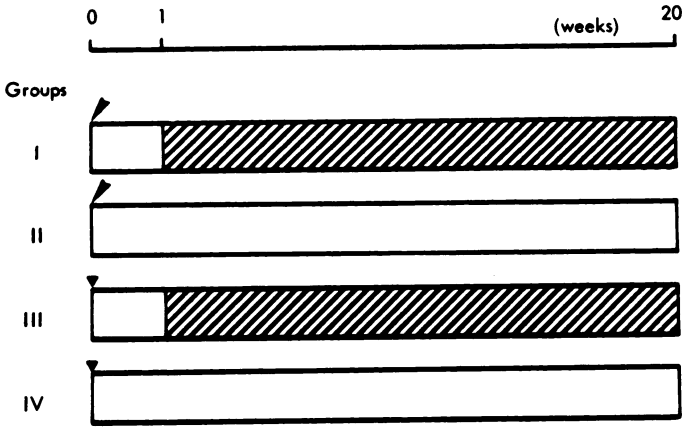

Chart 1. Experimental design. ▨, 2500, 500, 100, or 20 ppm PB in diet (see text); □, basal diet; ▶, single i.p. injection of DHPN, at 210 mg/100 g body weight for male rats and 420 mg/100 g body weight for female rats; ▼, single i.p. injection of 0.5 ml saline/100 g body weight.

PB or with PB alone. In contrast the groups treated with DHPN alone showed a decrease in body weight as compared to controls. The average weights of livers in all groups of female rats were similar, whereas PB-treated groups of male rats demonstrated an increase in liver weight; the increase was proportional to the dose of PB. The average thyroid weights showed a significant increase in all groups of rats, both male and female, treated with DHPN and PB or PB alone as compared to the nontreated control (Group IV).

Macroscopic Findings in Thyroid Tumors. The thyroids of rats in Group I-2 (DHPN followed by 500 ppm PB) had smooth, yellowish surfaces, whereas those of Group III-3 (100 ppm PB alone) had smooth, red-yellowish surfaces. Thyroids in Group I-2 were elastically firmer than those in Group III-3. Neither nodules and hemorrhage nor adhesion of thyroid to adjacent tissue were observed in any group.

Microscopic Findings in Thyroid Tumors. Using the classification criteria described by Napalkov (17), thyroid adenomas arising in the present experiment were divided into three types: (*a*) polymorphofollicular adenomas, which consisted of various sizes of follicles with lining cells exhibiting slightly basophilic abundant cytoplasm and large round nuclei; (*b*) microfollicular adenoma, characterized by small follicles with scanty colloid, the lining cells having moderately abundant cytoplasm and large round nuclei; and (*c*) papillary type adenoma, showing papillary proliferation of cells with basophilic abundant cytoplasm. No C-cell adenomas were found in the present experiment.

Thyroid carcinomas were of follicular type consisting of small or irregular follicles demonstrating papillary ingrowths or formations. The cells comprising these lesions had slightly basophilic cytoplasm and large nuclei with pale nucleoplasm. The histopathological characteristics of these thyroid tumors have been described in detail previously (5).

Nontumorous Thyroid Areas. Histological changes in nontumorous areas of thyroid tissue in each group are summarized in Table 1. Hyperplasia of thyroid follicles consisted of cells with large nuclei and clear abundant cytoplasm. Colloid decrease was evident in small follicles. A dose response with regard to PB-dependent development of hyperplasia, colloid decrease, and small follicles was observed. These histological changes were more remarkable in male rats than in female rats.

Table 1

Histological findings in nontumorous areas of the thyroids of Wistar rats treated with DHPN followed by different doses of PB

	Treatment		Hyperplasia		De-crease of colloid in folli-cles		Decrease in size of follicles	
Group	DHPN	PB (ppm)	M	F	M	F	M	F
I-1	Yes	2500	++[a]	++	++	+	+++	++
I-2	Yes	500	++	±	++	±	+++	+
I-3	Yes	100	±	±	±	±	±	+
I-4	Yes	20	±	±	±	±	±	±
II	Yes		±	±	±	−	±	−
III-1	No	2500	++	++	+	+	+++	++
III-2	No	500	++	±	+	±	+++	+
III-3	No	100	±	±	−	±	±	+
III-4	No	20	−	−	−	±	−	−
IV	No		−	−	−	−	−	−

[a] −, negative; ±, positive in a few rats; +, slight; ++, moderate; +++, marked.

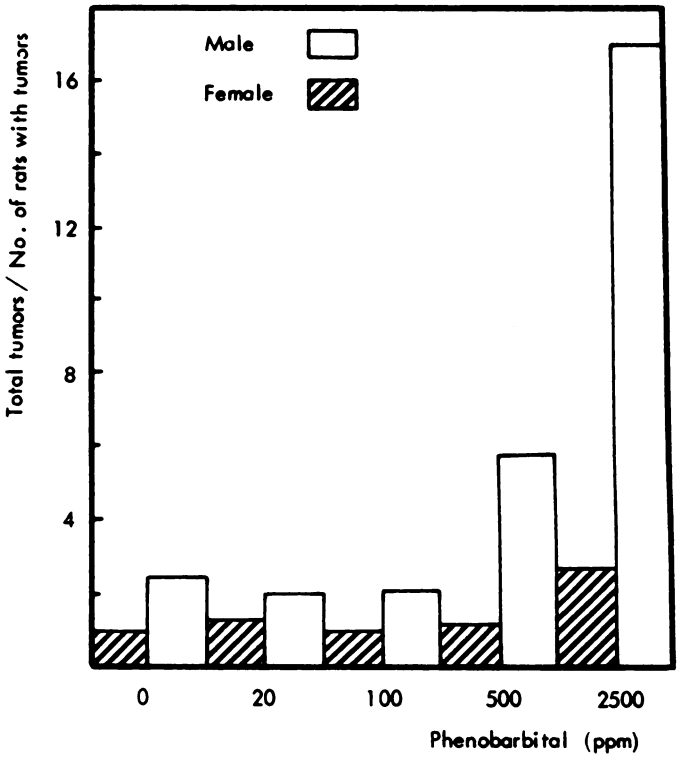

Chart 2. Sex difference in the induction of thyroid tumors in rats treated with DHPN followed by various doses of PB.

Sex Differential and Dose Dependence in Induction of Thyroid Tumors. Thyroid tumors, both adenomas and carcinomas of follicular or papillary type, were seen only in the animals treated with DHPN alone or followed by PB (Groups I-1 to I-4 and Group II). Chart 2 illustrates the sex difference with regard to induction of thyroid tumors. A single injection of DHPN resulted in production of approximately 1 tumor/female rat and 2.5 tumors/male rat. DHPN in combination with subsequent PB at a concentration as high as 500 ppm did not demonstrate an increase of tumor yield in female rats, in contrast to a 3-fold increase evident in male rats. Upon exposure of DHPN-treated

male rats to 2500 ppm of PB, a marked increase (approximately 8-fold) in tumor yield was observed. However, a <3-fold increase was seen in female rats similarly treated with DHPN and the highest dose of PB.

Tables 2 and 3 show the dose-dependent induction of thyroid tumors in male and female rats treated with DHPN followed by various doses of PB. A single injection of DHPN resulted in an 8% tumor incidence in both male and female rats. When the DHPN-treated rats were fed with basal diet containing 20, 100, 500, and 2500 ppm of PB, the incidences of follicular adenoma were, respectively, 8, 45, 70, and 66% in male rats and 12, 17, 50, and 58% in female rats. Development of papillary adenomas in male rats was seen only at the higher doses of PB, at 12 and 20%, respectively, for doses of 500 and 2500 ppm. In addition follicular carcinomas were seen in male animals treated with the higher dose of PB; they were 16 and 12% for 500 and 2500 ppm, respectively. Surprisingly neither follicular nor papillary carcinomas were induced in female rats; only a single papillary adenoma (low incidence of 4%) was yielded by PB posttreatment at a dose as high as 2500 ppm. No tumors of any type were observed in the control group (Group IV) or in rats treated with the various doses of PB alone (Groups III-1 to 4).

Table 2

Incidences of thyroid tumors in male Wistar rats treated with DHPN followed by different doses of PB

Group	Treatment: DHPN	Treatment: PB (ppm)	Effective no. of rats	Thyroid wt[a] (mg)	No. of rats with: Follicular (%): Adenoma	Follicular (%): Carcinoma	Papillary (%): Adenoma	Papillary (%): Carcinoma
I-1	Yes	2500	24	37.0 ± 9.0[b]	16[c] (66)	3 (12)	5 (20)	0
I-2	Yes	500	24	27.1 ± 6.9	17[c] (70)	4 (16)	3 (12)	0
I-3	Yes	100	24	21.8 ± 6.9	11[c] (45)	0	0	0
I-4	Yes	20	24	21.6 ± 3.5	2 (8)	0	0	0
II	Yes		24	22.5 ± 1.5	2 (8)	0	0	0
III-1	No	2500	24	22.3 ± 2.8	0	0	0	0
III-2	No	500	24	23.4 ± 2.3	0	0	0	0
III-3	No	100	24	20.2 ± 1.8	0	0	0	0
III-4	No	20	24	17.4 ± 1.6	0	0	0	0
IV	No		24	17.8 ± 0.9	0	0	0	0

[a] Means ± SD.
[b] Significantly different from Group II ($P < 0.05$, t test).
[c] Significantly different from Group II ($P < 0.05$, χ^2 test).

Table 3

Incidences of thyroid tumors in female Wistar rats treated with DHPN followed by different doses of PB

Group	Treatment: DHPN	Treatment: PB (ppm)	Effective no. of rats	Thyroid wt[a] (mg)	No. of rats with: Follicular (%): Adenoma	Follicular (%): Carcinoma	Papillary (%): Adenoma	Papillary (%): Carcinoma
I-1	Yes	2500	24	21.3 ± 3.6	14[b] (58)	0	1 (4)	0
I-2	Yes	500	24	22.5 ± 4.4[c]	12[b] (50)	0	0	0
I-3	Yes	100	23	16.7 ± 1.6	4 (17)	0	0	0
I-4	Yes	20	24	17.0 ± 2.7	3 (12)	0	0	0
II	Yes		24	17.1 ± 3.0	2 (8)	0	0	0
III-1	No	2500	24	19.0 ± 3.7	0	0	0	0
III-2	No	500	24	21.4 ± 1.1	0	0	0	0
III-3	No	100	24	22.5 ± 2.8[c]	0	0	0	0
III-4	No	20	24	17.4 ± 2.4	0	0	0	0
IV	No		24	16.6 ± 0.7	0	0	0	0

[a] Means ± SD.
[b] Significantly different from Group II ($P < 0.05$, χ^2 test).
[c] Significantly different from Group II ($P < 0.05$, t test).

DISCUSSION

In our previous work (5–8) we demonstrated that DHPN is a potent thyroid carcinogen in rats and that the induction of thyroid tumors by DHPN can be enhanced by posttreatment with PB (5, 7). The present paper documents our findings that a single dose of DHPN as an initiator and PB as a promoter results in thyroid tumor development. The enhancement of tumor production by PB in DHPN-initiated rats was dose dependent (Tables 2 and 3). The tumors induced under this experimental regimen were mainly follicular adenomas. Other types of thyroid tumors such as follicular carcinoma and papillary adenoma were observed only at the higher doses of PB (above 500 ppm). However, follicular carcinoma was observed only in male rats.

Interestingly there was a marked difference in tumor yield between male and female rats (Chart 2). PB at 500 ppm did not increase tumor yield in female rats, whereas there was an approximately 3-fold increase in male rats. PB at 2500 ppm increased tumor yield in male rats 8-fold, in contrast to <3-fold in female rats.

Two general approaches have been reported for inducing thyroid tumors in rats. The first approach aims at establishing a hormonal imbalance that will lead to tumor development. In this method the first stage in the development of thyroid tumors is inhibition of hormone production by thyroid tissue under the influence of antithyroid substances. The second stage is the sustained intensification of synthesis and release of TSH. This approach induces thyroid tumors selectively without tumors in other organs arising, but a long time is required to obtain tumors (21). The second approach is based on the use of strong chemical carcinogens. These substances, *e.g.*, DHPN, induce tumors not only in the thyroid gland but also in a wide spectrum of other organs (14). Further a combination of an antithyroid drug or goitrogen and chemical carcinogen can result in acceleration of tumor development in the thyroid. For example we demonstrated that giving DHPN as an initiating carcinogen and PB as a goitrogen efficiently induced thyroid tumors in rats (6, 7). Japundzic *et al.* (10, 11) had shown earlier that daily applications of PB for 90 days resulted in a marked increase in thyroid weight accompanied by hypertrophy and hyperplasia of the follicular cells, as well as disappearance of the intrafollicular colloid. The above effects of PB on thyroid tissue were found to be prevented by hypophysectomy or injection of exogenous thyroxin (12); in addition it was demonstrated that chronic administration of PB led to increased secretion of thyrotrophic hormone (12). It was suggested that the pituitary thyrotrophic hypersecretion induced by PB results either from thyroid hormone synthesis or from a changed peripheral metabolism of thyroid hormone (12). From these findings it was concluded that the goitrogenic effect of PB, unlike its growth-promoting influence on the liver, was secondary in nature and depended on an increased release of TSH from the pituitary gland.

The mechanisms by which PB promotes thyroid tumorigenesis are unknown. However, there is a possibility that PB, through activation of liver drug-metabolizing enzymes, could cause increased rates of thyroid hormone metabolism in the liver, which in turn could stimulate increased production of TSH by the

pituitary gland. On the other hand high doses of PB may escape the metabolic capacity of the liver and directly affect the pituitary or thyroid glands.

The present results indicate that the enhancement of thyroid tumor yield by PB may result from the "promotion" of the clones initiated by DHPN rather than a consequence of increases in the growth rates of thyroid tumors. To establish whether this possibility is correct, additional experiments involving multiple time points and an examination of the numbers and sizes of thyroid tumors are presently under way.

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