P-450 Enzyme Induction by 5-Ethyl-5-phenylhydantoin and 5,5-Diethylhydantoin, Analogues of Barbiturate Tumor Promoters Phenobarbital and Barbital, and Promotion of Liver and Thyroid Carcinogenesis Initiated by N-Nitrosodiethylamine in Rats

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ABSTRACT

Male F344/Ncr rats, 6 wk old, were fed 500 ppm of phenobarbital (PB) or equimolar doses of either 5-ethyl-5-phenylhydantoin (EPH) or 5,5-diethylhydantoin (EEH) in diet for 2 wk and hepatic cytochrome P-450-mediated alkoxyresorufin O-dealkylase and aminopyrine N-deethylation activities were determined. Both PB and EPH greatly increased P-450-mediated enzyme activities in rat liver while EEH was ineffective. To evaluate the hydantoins as tumor promoters, 5-wk-old male F344 rats were given a single i.p. injection of 75 mg N-nitrosodiethylamine/kg body weight. Beginning 2 wk later, they were placed either on normal diet or diet containing 500 ppm of PB or equimolar doses of EPH or EEH for the remaining experimental period. Control groups received an i.p. injection of saline followed by each of the test diets. Animals were sacrificed at either 52 or 78 wk. PB and EPH significantly enhanced the development of hepatocellular foci and hepatocellular adenomas at 52 wk and hepatocellular carcinomas at 78 wk in N-nitrosodiethylamine-initiated rats. Neither the incidence of hepatocellular neoplasms nor the number and size of hepatocellular foci was significantly increased by EEH. At 78 wk, both PB and EPH enhanced the development of thyroid follicular cell neoplasms in N-nitrosodiethylamine-initiated rats while no such enhancement was observed with EEH. Thus, EPH, a long-acting sedative/anticonvulsant, like the structurally similar PB, promoted hepatic cytochrome P-450-mediated activities in rat liver while EEH was ineffective.

INTRODUCTION

An increasing number of studies during the past 15 yr has shown that hepatocarcinogenesis is a multistep process, in which initiation and promotion stages have been identified in laboratory rats and mice (1–5). Since the original discovery (1) that hepatocarcinogenesis in rats could be promoted by PB, a variety of other drugs, insecticides, and some endogenous compounds such as steroid hormones have been shown to accelerate hepatocellular tumorigenesis in that species (6–10). The phenomenon of multistage hepatocarcinogenesis has been the subject of several recent reviews (11–13).

Our laboratory has been engaged in studies on structure-promoting activity relationships among sedative, anxiolytic, and anticonvulsant agents prescribed either singly or in combination for the treatment of neurological disorders. Because these drugs are often taken in relatively large doses on a daily basis for prolonged periods, they are realistic candidates for tumor-promoting agents. In recent studies (14–16) we have shown that certain of these compounds promote carcinogenesis in epithelia other than hepatocytes, including those of the thyroid follicle, renal tubule, and urothelium, and that liver tumor-promoting activity within a series of barbiturate derivatives varies greatly with molecular structure, persistence in tissues as measured by duration of sedative action, and efficiency of induction of specific monoxygenase enzyme activities.

Barbiturates share common structural features with several other classes of anticonvulsants including hydantoins, oxazolidinediones, and succinimides. Hydantoins, especially, are very similar to the barbituric acid derivatives in both chemical structure and pharmacological activities. For example, PB and N-nitrosodimethylamine in Rats

MATERIALS AND METHODS

Chemicals

PB (M, 232,000; purity >99%) was purchased from Sigma Chemical Company, St. Louis, MO. EPH (M, 204,000) and EEH (M, 156,000)
No extraneous NMR peaks were observed in solutions of either compound. 7-Ethoxy-, 7-pentoxy, and 7-benzyloxyresorufin were purchased from Molecular Probes, Inc., Junction City, OR. Dicumarol, resorufin, and aminopyrine were from Aldrich Chemical Company, Milwaukee, WI, and fluorescamine from Roche Diagnostics, Nutley, NJ. DEN (Sigma) was analyzed by gas-liquid chromatography and its purity was found to exceed 99%.

Tumor Promotion Study

Two hundred forty weanling male F344/NCr rats were obtained from Animal Production, National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD. All animals were housed in an American Association for Accreditation of Laboratory Animal Care-accredited facility. They were housed four per cage in autoclavable poly-carbonate cages and given food (NIH autoclavable formula 31 modified to contain 6% fat) and water ad libitum. The animals were maintained at a temperature of 68 to 72°F and a relative humidity of 50 ± 5% with 12 changes room air per h. At 5 wk of age, they were randomly divided into 8 groups of 30 rats each. Rats in carcinogen-treated groups received 12 changes room air per h. At 5 wk of age, they were randomly divided into 8 groups of 30 rats each. Rats in carcinogen-treated groups received 5.5-diethylhydantoin

**Fig. 1.** Structures of 5,5-disubstituted barbiturates and hydantoins.
decrease in body weight gain in older rats (78 wk) receiving either PB (409 ± 85 g, mean ± SD) or EPH (415 ± 68 g) following DEN treatment was related to higher incidence of malignant hepatocellular neoplasms as compared to those that received DEN alone (425 ± 43 g). A significant increase in the liver to body weight ratio was observed at 52 and 78 wk of age in rats that received either PB or EPH following DEN administration, in comparison with rats that had been given DEN alone. This increase in liver weight in PB- and EPH-treated groups was clearly due to the growth of neoplasms in these animals. No significant increase in liver weights or liver:body weight ratio was observed in rats fed EEH following exposure to DEN (group 4).

Survival. All rats in every treatment group were alive at 52 wk of age. Between 52 and 78 wk survival of DEN-EEH (group 4) rats was better than that of DEN-EPH (group 3) or DEN-PB (group 2) rats (refer to Fig. 2). In the DEN-EPH group 8 of 15 rats (53%) and in the DEN-PB group 9 of 15 rats (60%) survived until 78 wk. All of the rats that died between 52 and 78 wk had hepatocellular lesions. All rats given hydantoins or PB but no DEN or left untreated (groups 5–8) survived for the duration of this study.

Development of Hepatocellular Foci. At 52 wk, foci of altered hepatocytes of clear, eosinophilic, mixed, and basophilic cell types were seen in animals treated with either DEN alone (group 1) or DEN followed by the test chemicals. Mixed and basophilic foci were more commonly observed in animals treated with DEN alone whereas eosinophilic and clear cell foci were especially evident in DEN-PB (group 2) or DEN-EPH (group 3) animals.

As shown in Fig. 3, animals that were initiated with DEN and then given either PB (group 2) or EPH (group 3) developed significantly more foci/cm² than did animals treated with DEN alone (group 1). Although the number of DEN-initiated foci (group 1) was slightly increased after EEH treatment (group 4), this difference was not significantly different. No foci were observed at this time (52 wk) in control animals including those given only PB, EPH, or EEH (groups 5–8).

Fig. 4 depicts the average size of foci in different groups of rats at 52 wk of age. The mean volume of foci was significantly higher (P < 0.05) in groups given PB (group 2) or EPH (group 3) following DEN initiation than in rats given only DEN (group 1). The mean volume of foci in the rats given EEH after DEN (0.13 ± 0.17, group 4) was significantly lower (P < 0.05) than in those given either PB (group 2) or EPH (group 3) after DEN administration.

Development of Hepatocellular Neoplasms. The incidences of hepatocellular tumors observed in different experimental groups at 52 wk are shown in Table 1. In rats initiated with DEN, both PB and EPH enhanced the incidence of hepatocellular adenomas. EPH was as effective as PB in promoting hepatocarcinogenesis initiated by DEN. Also, histologically, the liver architecture (i.e., enlargement of centrilobular hepatocytes) of rats treated with EPH did not differ from hepatic morphology in rats treated with PB. Rats of both groups had hepatocytomegaly. The incidence of hepatocellular carcinomas was 10% in DEN-PB animals and 20% in DEN-EPH animals. EEH treatment subsequent to DEN administration (group 4) did not significantly modify the incidence of hepatocellular neoplasms and the livers of EEH-fed rats were not significantly different than those of rats given only DEN. Results presented in Table 1 clearly show that group 2 animals treated with PB subsequent to DEN and group 3 animals receiving EPH following DEN had significantly enhanced hepatocellular adenoma yields compared to rats treated with DEN alone (P < 0.05).

By 78 wk, almost all of the DEN-treated rats (groups 1–4) had developed hepatocellular adenomas although the multiplicity of such lesions was significantly greater (P < 0.05) in group 2 (DEN-PB) and group 3 (DEN-EPH) rats as compared to group 1 animals (DEN alone; Table 1). The incidence of hepatocellular carcinomas (Table 1) was also significantly higher (P < 0.05) in rats given either PB (group 2, 70%) or EPH (group 3, 60%) subsequent to DEN. Multiple hepatocellular carcino-
TUMOR PROMOTION BY HYDANTOINS

Table 1 Effect of PB, EPH, and EEH on hepatocellular carcinogenesis initiated by DEN in male F344/NCr rats

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Hepatocellular tumors observed at 52 wk*</th>
<th>Between 52 and 78 wk*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of animals with hepatocellular tumors</td>
<td>Tumors per rat with tumors</td>
</tr>
<tr>
<td></td>
<td>Adenomas (%)</td>
<td>Carcinomas (%)</td>
</tr>
<tr>
<td>DEN</td>
<td>3 (20)</td>
<td>0</td>
</tr>
<tr>
<td>DEN-PB</td>
<td>14 (93)*</td>
<td>1 (7)</td>
</tr>
<tr>
<td>DEN-EPH</td>
<td>13 (87)*</td>
<td>4 (27)</td>
</tr>
<tr>
<td>DEN-EEH</td>
<td>2 (13)</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Saline-PB</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Saline-EPH</td>
<td>1 (7)</td>
<td>0</td>
</tr>
<tr>
<td>Saline-EEH</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* There were 15 rats in each group.
* Statistically significant compared to group 1 (DEN); P < 0.05.

Table 2 Effects of hydantoins administered in the diet on cytochrome(s) P-450 activities in the rat

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>O-Dealkylation of phenoxazone ethers*</th>
<th>Aminopyrine N-demethylation†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>56.3 ± 9.2</td>
<td>31.9 ± 5.7</td>
</tr>
<tr>
<td>PB</td>
<td>122.0 ± 12.7*</td>
<td>947.0 ± 15.0*</td>
</tr>
<tr>
<td>EPH</td>
<td>123.0 ± 13.6*</td>
<td>1062.0 ± 43.0*</td>
</tr>
<tr>
<td>EEH</td>
<td>87.0 ± 18.0</td>
<td>81.0 ± 6.8</td>
</tr>
</tbody>
</table>

* F344 rats received control diet or diet containing either 500 ppm phenobarbital or equimolar doses of ethylphenylhydantoin or diethylhydantoin for 15 days. Enzyme assays were done in duplicate; values given are mean ± SD for five male rats per treatment.
* Values are in units of picoleses resorufin formed/min/mg S-9 protein at 28°C.
† Values are in units of nanomoles formaldehyde formed/min/mg microsomal protein at 37°C.
* Significantly different from control group, P < 0.05 (2-sample Kruskal-Wallis nonparametric rank test).

Hepatocellular carcinomas were commonly observed in rats of groups 2 and 3 while few such lesions were seen in animals of groups 1 and 4 (Table 1). Furthermore, two rats in group 2 (DEN-PB) and three rats in group 3 (DEN-EPH) had lung metastases (Figs. 5 and 6) while none was found in groups exposed to DEN alone (group 1) or DEN followed by EEH (group 4).

Development of Nonhepatic Tumors. The most common types of nonhepatic tumors observed in this study included those of the nasal cavity, thyroid, pituitary gland, testes, and lymphoreticular system (Table 2). Nasal cavity tumors were observed only in DEN-treated rats and their incidence was not affected by the administration of test compounds. The incidences of testicular tumors (Leydig cell tumor), pituitary tumors, and large granular lymphocyte leukemias were predictably high in older rats (78 wk) of both control and experimental groups and were unaffected by treatment.

Thyroid tumors were most frequently observed in rats of groups 2 (DEN-PB) and group 3 (DEN-EPH). Thyroid tumors of follicular cell origin were seen in 5 of 15 rats (33%) in group 2 and 4 of 15 rats (27%) in group 3 while no such tumors were observed in other groups. Histologically, these tumors were classified as follicular cell adenomas (Fig. 7) and adenocarcinomas. The historical incidence of thyroid tumors in rats given DEN alone in our two last studies (14, 16) was 2 tumors in 35 animals. Thus, PB and EPH but not EEH have a significant (P < 0.05) enhancing effect on the development of thyroid tumors in DEN-initiated animals. Although follicular cell hyperplasias were seen in PB only or EPH only animals no goitrogenic changes were observed.

Biochemical Study

Effects of PB, EPH, and EEH on Body and Liver Weights. Animals in all treatment groups showed normal and compa-
able weight gains. Ratios of liver to body weights following 15 days dietary administration were significantly higher \((P < 0.05)\) in rats fed EPH \((7.09 \pm 0.40, \text{mean} \pm \text{SD})\) and PB \((7.53 \pm 0.29)\) as compared to those fed EEH \((5.86 \pm 0.46)\) or control diet \((5.65 \pm 0.23)\). Absolute weights were also significantly increased in EPH- and PB-treated rats compared to the controls.

Effects of PB, EPH, and EEH on Ethoxy-, Pentoxy-, and Benzyloxyresorufin O-Dealkylase Activities. As shown in Table 2, both PB and EPH dramatically (~30-fold) increased pentoxy- and benzyloxyresorufin O-dealkylase activities in rat liver after 15 days of feeding. On the other hand, only a slight (2- to 3-fold) increase in activity of these enzymes was evident in livers of rats fed EEH. Ethoxyresorufin O-dealkylase activity was induced about 2- to 3-fold over control values by PB and EPH while no such increase in the activity of this enzyme was seen following EEH treatment.

Effects of PB, EPH, and EEH on Aminopyrine \(N\)-Demethylase Activity. A significant increase of aminopyrine \(N\)-demethylase activity over control values was observed in rats fed all test compounds (Table 2). However, the increase was much higher in rats fed either EPH or PB (3- to 4-fold) than in those fed EEH (1.5-fold).

**DISCUSSION**

EPH is a hepatic metabolite of the anticonvulsant drug 3-methyl-5-phenyl-5-ethylhydantoin \((17)\) and has been considered to contribute to both the therapeutic effect and the toxicity of the parent drug. At equal plasma concentrations, EPH and PB produce similar anticonvulsive effects \((17)\). Under the trade name Nirvanol, EPH was used clinically for the treatment of chorea in children in the 1920s but caused a high incidence of toxic side effects.

From this study it is clear that EPH is also comparable to PB in both the specificity and the intensity of its inducing effect on hepatic \(O\)-dealkylase activities toward the phenoxazone substrates pentoxy- and benzyloxyresorufin, both of which are specifically mediated in rats by the PB-inducible form of cytochrome P-450, P-450b \((32)\). Both PB and EPH cause increases in these activities in rats of approximately 30- to 35-fold. In comparison with the 2- to 4-fold increases in aminopyrine \(N\)-demethylase also induced by these compounds, the alkoxyresorufin \(O\)-dealkylase reactions clearly provide an extremely sensitive assay for P-450, induction.

This study clearly showed that EPH, given after the initiating dose of DEN, like its barbiturate analogue PB, also had an enhancing effect on the development of hepatocellular foci and hepatocellular neoplasms. There was a significant increase \((P < 0.05)\) in the number of focus transactions/cm\(^2\) in livers of rats exposed to EPH as compared to the number in livers of rats exposed to DEN alone (Fig. 3). EPH also enhanced the development of hepatocellular neoplasms resulting in a significant increase in the percentage of rats bearing liver tumors and in the number of tumors per liver (Table 1). At equimolar doses, both PB and EPH exhibited approximately equal potency in enhancing liver growth and promoting preneoplastic and neoplastic liver lesions in DEN-pretreated rats. Moreover, a promoting effect on the incidence of follicular tumors of the thyroid, comparable in magnitude to that of PB \((14)\), was observed in rats that received EPH following DEN initiation. EPH thus appears fully comparable to PB in regard to both the epithelia on which it has promotion effects and the dosage required for promotion in these tissues.

EEH is structurally similar to barbital. However, unlike barbital, EEH is pharmacologically inactive as either a hypnotic or an anticonvulsant \((36)\). Barbital has been shown to promote hepatocarcinogenesis \((14, 20)\) and thyroid and renal carcinogenesis \((14, 15)\) in rodents, and is also known to induce hepatic cytochrome P-450 \((14)\), PB-specific cytochrome(s) P-450 activities \((24)\), and aminopyrine \(N\)-demethylase activity \((14)\) in rats. In contrast, EEH failed to increase liver growth or promote development of either preneoplastic or neoplastic hepatocellular lesions and caused minimal increase in cytochrome P-450-dependent drug-metabolizing enzymes.

Several recent reports have shown a strong correlation between the liver tumor-promoting activity of several distinct classes of compounds and their ability to induce liver hypertrophy and cytochrome(s) P-450-dependent drug-metabolizing enzymes \((8, 19, 24)\). The metabolism of many substances is mediated by specific forms of cytochrome P-450. Thus, the dealkylation of pentoxy- and benzyloxyresorufin is mediated by the major PB-inducible forms of cytochrome(s) P-450 in the rat \((29, 32, 33)\). Within the class of barbiturates, the ability to promote hepatocarcinogenesis is closely associated with the ability of each compound to increase liver growth and induce dealkylase activities mediated by the major PB-specific cytochrome P-450 (pentoxy- and benzyloxyresorufin \(O\)-dealkylase) in male rats \((16, 24)\). The correlation between induction of alkoxyresorufin dealkylase activity and capacity to promote hepatocarcinogenesis previously established for barbiturates clearly extends to their hydantoin analogues. Thus, the induction of liver growth associated with functional increases in the major PB-inducible forms of cytochrome(s) P-450 is consistent with this view. Interestingly, in our recent study, we found that the liver tumor-promoting chlorinated hydrocarbon pesticides \((e.g., \text{DDT}, \text{lindane})\) are also potent inducers of pentoxy- and benzyloxyresorufin \(O\)-dealkylase activities in rats.\(^5\) Thus, the ability to induce PB-
specific cytotoxic(s) P-450 in the liver appears to warrant further evaluation as a molecular marker for screening chemicals for ability to promote hepatocarcinogenesis.

The commonly used anticonvulsant 5,5-diphenylhydantoin (phenytoin; Dilantin) was found by Peraino et al. (19) to be ineffective in promoting hepatocellular carcinogenesis in Sprague-Dawley rats when fed mixed in diet at 500 ppm. These authors also observed no increase in liver weight or liver DNA synthesis in diphenylhydantoin-treated animals in their acute exposure study. Phenytoin has, however, been shown to induce hepatic DNA synthesis and hyperplasia in the rat in an earlier study (37). This drug is known to induce microsomal enzyme activity in several rodent species (38, 39) and in man (40, 41). We have found this drug to be effective in inducing O-dealkylation of pentoxyc- and benzyloxyresorufin in F344 rats, although the magnitude of the response is approximately one-half of that achieved with EPH or PB. Thus, based on our results and those of others (37, 38, 41), we consider that further experiments are needed on the possible effects of diphenylhydantoin on hepatocarcinogenesis in rats.

The mechanisms underlying the promoting action of EPH or of the barbiturates in the rat liver are not yet known. However, the present study clearly showed that EPH exerted biochemical effects quite similar to those of PB, a well-established rodent liver tumor promoter. Thus, in consideration of similarities in the biochemical effects between PB and EPH, it seems probable that the action of EPH in liver tumor promotion is quite similar to that of PB. However, the present data do not rule out the possibility that tumor promotion by PB and by EPH proceeds by different mechanisms.

REFERENCES


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