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Sex-dependent promoting effect of polychlorinated biphenyls on enzyme-altered islands induced by diethylnitrosamine in rat liver

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Abstract

The promoting effect of Clophen A 50, a commercial mixture of polychlorinated biphenyls (PCBs) on preneoplastic islands, initiated by diethylnitrosamine (DEN), was studied in male and female Sprague-Dawley rats. The islands were identified histochemically by loss of adenosine-5′-triphosphatase (ATPase) and/or emergence of gamma-glutamyltranspeptidase (GGTase). Treatment with 12 x 8 mg DEN/kg body wt./day initiated a similar number and total area of islands in males and females. Additional weekly application of Clophen A 50 (50 or 100 mg/kg body wt./week, for 7 weeks) enhanced the number of ATPase-deficient islands 3-fold in males and 9-fold in females. The total area was increased 4-fold in males and 15-fold in females. Number and area of GGTase-positive islands were similarly enhanced. The emergence of a small number of islands after application of Clophen A 50 alone may indicate a weak carcinogenic potency. PCB treatment caused an increase in liver weight, which amounted to ~55% in males and 20% in females compared to controls. This increase is partly due to cell hypertrophy, as indicated by determination of cell size. The mitogenic activity of Clophen A 50 was evaluated by measurement of the mitotic index of unaltered hepatocytes at 24, 48 h, and 7 days after application of a single dose (100/mg/kg body wt.) of Clophen A 50. The mitotic index in control animals of both sexes was ~0.3%, and was enhanced ~8-fold in males, 24 h after PCB treatment. In females only a slight, non-significant increase was observed. The results indicate that the sex-dependent promoting effect of Clophen A 50 is independent from its mitogenic action.

Introduction

Various drugs and environmental chemicals favour the formation of tumors and preneoplastic lesions in the livers of rodents (for review see 1). In livers of rats, pretreated with carcinogens, number and area of enzyme-altered, preneoplastic islands are markedly enhanced by administration of phenobarbital (2–4), alpha-hexachlorocyclohexane (5), and polychlorinated biphenyls (PCBs)* (6–8). These substances are mitogenic and cause liver enlargement (9) by cell hypertrophy and hyperplasia (5), while non-promoting agents, e.g. 3-methylcholanthrene, amobarbital and diphenylhydantoin, do not enhance liver weight (10).

We observed that PCBs, which are potent liver tumor promoters (11), produce a higher increase in liver weight in male rats than in females. Therefore, it seemed to be a valuable model for studying whether a relationship between tumor promotion and liver growth exists. Recently, a higher sensitivity of female rats to promotion of gamma-glutamyltranspeptidase (GGTase)-positive islands by hexachlorobenzene and lindane has been described (12).

The present study demonstrates the promoting effect of PCB-treatment on adenosine-5′-triphosphatase (ATPase)-deficient and GGTase-positive islands, initiated by diethylnitrosamine (DEN) in male and female Sprague-Dawley rats. Hypertrophy of hepatocytes was evaluated by an indirect measurement of cell size. The mitotic index was determined in normal liver after application of a single dose of PCBs, in order to examine whether cell hypertrophy and hyperplasia coincide with the promoting effect.

Materials and Methods

Animals

Male and female Sprague-Dawley rats (inbred strain, Neuherberg, FRG), 6 weeks of age, were used. Two animals were housed together in Macrolon cages. They received a standard pellet diet (Altromin 1320, Lage, FRG) and drinking water *ad libitum*.

Chemicals

DEN was obtained from p.A., Serva, (Heidelberg, FRG). Clophen A 50, Bayer, (Leverkusen, FRG), is a technical mixture of PCBs. The main components are pentachlorobiphenyls (44%), tetrachlorobiphenyls (28%) and hexachlorobiphenyls (16%). The mean chlorine content amounts to ~54% (Wrabetz, personal communication, 1979).

Dosing schedule

DEN was dissolved in water (2 mg/ml) immediately before use. Clophen A 50 was dissolved in olive oil (25 or 50 mg/ml) and stored at 4°C. Male and female rats were separated into three groups of four rats each. All groups received 8 mg DEN/kg body wt./day for 12 consecutive days by gastric intubation. One week after cessation of nitrosamine application, two groups of males and females were treated with 50 or 100 mg Clophen A 50/kg body wt. once weekly for seven consecutive weeks.

One group of males and females received 100 mg Clophen A 50/kg body wt. only. Controls were treated with olive oil.

Histochemistry

The animals were sacrificed by cervical dislocation 12 weeks after beginning the experiment. Serial cryostat sections, 8 μm thick, were prepared from two liver lobes and stained for ATPase, according to Wachstein *et al.* (13) and GGTase, according to Lojda *et al.* (14), for details see Deml *et al.* (15). Of eight sections, a total area of 8 cm² was evaluated for ATPase and 4 cm² for GGTase. Number and area of enzyme-altered islands were determined, using a semiautomatic picture analyzer (Videoplan, Kontron, Eching, FRG).

Evaluation of cell size

For an estimation of cell enlargement, the number of hepatocytes in a defined area was counted, according to Bannasch *et al.* (16). A decrease in average cell number per area indicates an increase in cell size. Cryostat sections from the animals, treated with DEN or DEN and 50 mg Clophen A 50/kg body wt. were stained with Weigerts hemalaun. The nuclei of hepatocytes were counted at 630 x magnification in 100 fields (0.0154 mm²/field), corresponding to 2000–2500 cells/animal.

Determination of mitotic index

Male and female Sprague-Dawley rats, 12 of each sex, received a single dose of 100 mg/kg body wt. Clophen A 50 at 8.00 a.m. Control rats, six of each sex, received olive oil only. Groups of four animals each were killed 24 and 48 h, and 7 days after PCB treatment. Control rats were killed at the beginning of the experiment and after 24 h, 48 h, and 7 days. Fifteen hours prior to sacrifice, a single i.p. dose of 0.1 mg/kg body wt. colchicine was given. Pieces of liver were fixed in collidine-buffered formaldehyde, dehydrated in ethanol and embedded in hydroxyethylmethacrylate (EFL-67,

*Abbreviations: ATPase, adenosine-5′-triphosphatase (EC 3.6.1.3.); GGTase, gamma-glutamyltranspeptidase (EC 2.3.2.2.); DEN, diethylnitrosamine; PCBs, polychlorinated biphenyls.

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Serva, Heidelberg, FRG). The sections, 2 μm thick, were stained with Weigerts hemalaun. The number of late prophases and metaphases was measured in four sections from each animal. Two hundred and fifty fields, corresponding to ~25 000 cells/animal were evaluated at a magnification of 400 x. The number of cells was counted in five fields/animal.

Statistical evaluation was performed by a two-sided u-test which is suited for statistical analysis without the prerequisite of gaussian distribution.

Results

Body and liver weight

Body weight was not affected by PCB treatment. Liver weight and liver-to-body weight ratio were significantly increased in all groups treated with Clophen A 50 (Table I). No difference was seen between the groups treated with 50 or 100 mg/kg body wt. The increase of liver weight was ~55% in male and 20% in female rats, compared to DEN-treated animals.

Effect of PCBs on enzyme-altered islands

ATPase-deficient islands. Area and number of ATPase-deficient islands following treatment with 12 x 8 mg DEN/kg body wt./day were similar in male and female rats (Table II). Additional treatment with 50 mg Clophen A 50/kg body wt. enhanced the number of islands ~3-fold in males and 9-fold in females (Table II). The total island area was enhanced 4-fold in males and 15-fold in females. The average island size increased to ~130% in males and 160% in females, compared to DEN-treated animals. The number and area of islands in the animals treated with 100 mg Clophen A 50/kg were in the same order of magnitude as in those treated with 50 mg/kg. Only a few islands (0.5 – 2 per cm²) emerged when 100 mg Clophen A 50/kg body wt./week were given alone (Table II). In control rats, treated with olive oil, no enzyme-altered islands were observed.

GGTase-positive islands. DEN initiated only a few GGTase-positive islands (Table II). The total area amounted to 3% of the area of ATPase-deficient islands in males and 14% in females. Additional application of 50 mg Clophen A 50/kg body wt. enhanced the number 6-fold in males and 13-fold in females, while the total island area was increased to 26-fold and 30-fold, respectively. Treatment with 100 mg Clophen A 50/kg body wt. resulted in a greater increase in total island area (~90-fold) in females only. With DEN, the percentage of islands showing coincident loss of ATPase and emergence of GGTase amounted to ~7% in males and 13% in females. In the groups treated with DEN plus 50 and 100 mg Clophen A 50, the percentage of these islands was enhanced to 48% and 44% in males and 65% and 79% in females, respectively.

Cell hypertrophy

The number of cells per area was similar in male and female rats, treated with DEN, and likewise in both sexes, treated with DEN and Clophen A 50 (Table III). In contrast, a significant decrease of cell number was observed in males (27%) as well as in females (25%), treated with DEN and Clophen A 50, compared to the corresponding group treated with DEN only.

Mitogenic effect of Clophen A 50

In male and female control animals, the mitotic rate amounted to ~0.3%. The values of control animals killed at different times are summarized since no time-dependent difference was observed (Table IV). Twenty four hours after application of a single dose of 100 mg Clophen A 50/kg body wt., the mitotic rate was enhanced ~8-fold in males, while in

Table I. Liver and body weight in male and female Sprague-Dawley rats, treated with 12 x 8 mg DEN/kg body wt./day and 50 (100) mg Clophen A 50/kg body wt./week, for 7 weeks.

	Liver weight	Body weight	Liver weight as % body weight
Males			
DEN	11.0 ± 0.9[a]	445 ± 23	2.5 ± 0.1
DEN/ 50 mg A50/kg	17.0 ± 1.0[b]	454 ± 24	3.8 ± 0.1[b]
DEN/100 mg A50/kg	17.1 ± 0.9[b]	443 ± 12	3.9 ± 0.2[b]
100 mg A50/kg	15.9 ± 0.7[b]	486 ± 14	3.3 ± 0.2[b]
Control	11.4 ± 0.6	438 ± 17	2.6 ± 0.1
Females			
DEN	7.7 ± 0.4	279 ± 6	2.8 ± 0.1
DEN/ 50 mg A50/kg	9.4 ± 0.8[b]	264 ± 12	3.6 ± 0.2[b]
DEN/100 mg A50/kg	9.7 ± 1.0[b]	265 ± 14	3.6 ± 0.2[b]
100 mg A50/kg	9.5 ± 0.7[b]	266 ± 10	3.6 ± 0.2[b]
Control	7.7 ± 0.4	257 ± 16	2.9 ± 0.3

[a]Mean ± S.D. of 4 animals. [b]Significantly different from DEN-treated animals, (u-test, $p = 0.01$).

Table II. Number and area of ATPase-deficient (−) and of GGTase-positive (+) islands in male and female Sprague-Dawley rats, treated with 12 x 8 mg DEN/kg body wt./day and 50 (100) mg Clophen A 50/kg body wt./week for 7 weeks.

	ATPase (−)		GGTase (+)	
	number no./cm²	area mm²	number no./cm²	area mm²/cm²
Males				
DEN	18 ± 5[a]	0.17 ± 0.05	5 ± 2	0.005 ± 0.002
DEN/ 50 mg A50/kg	61 ± 9[b]	0.74 ± 0.13[b]	28 ± 5[b]	0.13 ± 0.03[b]
DEN/100 mg A50/kg	86 ± 5[b]	0.73 ± 0.01[b]	28 ± 7[b]	0.17 ± 0.05[b]
100 mg A50/kg	0.5 ± 0.1	0.005 ± 0.001	n.d.[c]	n.d.
Control	0.0	0.0	n.d.	n.d.
Females				
DEN	14 ± 8	0.14 ± 0.07	6 ± 2	0.02 ± 0.01
DEN/ 50 mg A50/kg	118 ± 34[b,c]	2.15 ± 0.66[b,c]	82 ± 26[b,c]	0.62 ± 0.23[b,c]
DEN/100 mg A50/kg	125 ± 42[b,c]	2.44 ± 1.50[b,c]	89 ± 19[b,c]	1.72 ± 1.00[b,c]
100 mg A50/kg	2 ± 0.5[c]	0.05 ± 0.04[c]	2 ± 1	0.006 ± 0.007
Control	0.0	0.0	n.d.	n.d.

[a]Mean ± S.D. of 4 animals. [b]Significantly different from DEN-treated animals (u-test, $p = 0.01$). [c]Significantly different from the corresponding group of male rats ($p = 0.01$). [d]n.d. = not determined.

Table III. Number of hepatocytes per field (0.0154 mm²) in male and female Sprague-Dawley rats treated with DEN and 50 mg Clophen A 50/kg body wt.[a]

	Males	Females
DEN	25.5 ± 4.9[c]	25.5 ± 5.3
DEN/A50	18.6 ± 4.4[c]	19.2 ± 4.6[c]

[a]For experimental details see **Materials and Methods**. [b]Mean ± S.D., n = 400 fields. [c]Significantly different from the group treated with DEN (u-test, $p = 0.01$).

Table IV. Mitotic index (%) of hepatocytes in livers of male and female Sprague-Dawley rats treated with a single dose of 100 mg Clophen A 50/kg body wt. at different times after the application[a].

	Mitotic index (%)			
	Control (untreated)	Time after application of Clophen A 50		
		24 h	48 h	7 days
Males	0.36 ± 0.08[b]	2.30 ± 1.40[c]	0.56 ± 0.14	0.40 ± 0.24
Females	0.27 ± 0.12	0.38 ± 0.23	0.22 ± 0.15	0.13 ± 0.05

[a]For experimental details see **Materials and Methods**. [b]Mean ± S.D. of 6 animals in the control groups, and 4 animals in the groups treated with Clophen A 50. [c]Significantly different from control, (u-test, $p = 0.01$).

females only a slight, non-significant increase was observed. Forty eight hours and 7 days after PCB treatment, the mitotic rate was in the same range of magnitude as in controls.

Discussion

The promoting effect of Clophen A 50 on preneoplastic islands in rat liver is sex-dependent. Female rats are more susceptible to promotion by PCBs, as indicated by a higher rate of increase in number and area of ATPase-deficient and GGTase-positive islands (Table II). The higher dose (100 mg/kg) of Clophen A 50 resulted in an additional increase in area of GGTase-positive island in females only. In controls, no islands were found. In the groups treated with Clophen A 50 only, a very small number of islands was observed. Thus, a weak carcinogenic activity of Clophen A 50 cannot be excluded.

A comparable sex-dependent difference in promotion of GGTase-positive islands has been demonstrated for hexachlorobenzene and lindane (12). The authors suggested that the different susceptibility may either by caused by the intrinsic estrogenic activity of these substances or by induction of estrogen metabolism, since some estrogen hormones (17,18) and chemicals with estrogenic activity, e.g. DDT (10), act as promoters in hepatocarcinogenesis.

PCBs enhance steroid hormone metabolism (19,20) and have an estrogenic effect in female rats (21,22). Therefore Clophen A 50 may act similarly either by induction of steroid metabolism or by intrinsic estrogenic activity.

In contrast to the increase in island number and area, the increase in liver weight was higher in males than in females. Enlargement of liver has been reported for various liver tumor promoters (5,9), while non-promoting agents did not affect liver weight (10). Therefore, it has been suggested that an increase in number and area of hyperplastic nodules due to treatment with promoting agents may be associated with a stimulation of liver growth (9). Liver enlargement is caused by cell hypertrophy and hyperplasia (23). Our measurements demonstrate cell hypertrophy due to PCB treatment in male rats as well as in females (Table III). Cell size and the rate of increase, indicated by a decrease in number of hepatocytes per area was equal in males and females.

A higher rate of proliferation in GGTase-positive cells compared to unaltered hepatocytes was observed in DEN-treated female Wistar rats (5). The mitotic activity of unaltered as well as GGTase-positive hepatocytes was markedly enhanced when the rats were treated additionally with a single dose of the tumor promoters alpha-hexachlorocyclohexane or cyproterone acetate. This finding supports the suggestion that liver cell proliferation coincides with the promoting effect.

In the present work, the mitogenic activity of Clophen A 50 was determined in normal liver tissue. The mitotic index was measured after application of a single non-necrogenic dose of Clophen A 50, corresponding to the higher dose used in the promotion experiment. It was significantly enhanced in males, but not in females (Table IV). This suggests that the different rate of increase in liver weight is caused mainly by cell hyperplasia. The influence of Clophen A 50 on the mitotic rate of cells in enzyme-altered islands of male and female rats is presently under investigation.

In conclusion, growth of enzyme-altered islands is not correlated with the mitogenic activity of Clophen A 50. The results indicate that the promoting effect of Clophen A 50 is not necessarily associated with the stimulation of liver growth.

Further study of the sex-dependent difference in promotion may give some insight into the mechanism of promotion by PCBs, which is still unknown (1). In addition, the data indicate that female rats are highly suitable for testing the promoting activity of drugs and environmental chemicals.

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