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Susceptibility of Rat Preneoplastic Hepatocytes to Liver-tumor Promoters: Effects of Phenobarbital and Ethyl-α-*p*-chlorophenoxyisobutyrate on Phalloidin Sensitivity

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SUMMARY

The effects of the liver-tumor promoters phenobarbital (PB) and ethyl-α-*p*-chlorophenoxyisobutyrate (CPIB) on phalloidin sensitivity of normal and preneoplastic hepatocytes were examined. Hepatocytes were isolated by a collagenase perfusion method from rats fed either 0.05% PB or 0.25% CPIB for 14 weeks, and from those treated for 12 weeks with these agents followed by 2-week withdrawal of the agents. Preneoplastic hepatocytes were isolated from enucleated nodules induced by the Solt-Farber method in the livers of rats, either treated or not treated with PB or CPIB.

The phalloidin sensitivity of hepatocytes of the rats treated with PB or CPIB deceased, showing 73 to 90% of the control value in the former and 88 to 97% in the latter, depending on the concentration of phalloidin. After the 2-week withdrawal of the agents, the sensitivity recovered completely. The sensitivity of the preneoplastic hepatocytes of rats treated with PB or CPIB were far less sensitive to phalloidin than the untreated preneoplastic cells. The sensitivity was 28 to 54% in the preneoplastic cells of PB-treated and 54 to 59% in the cells of CPIB-treated rats compared to the values in the untreated preneoplastic cells. After the 2-week withdrawal of PB or CPIB, the sensitivity increased but was not completely recovered. Thus, preneoplastic hepatocytes were more susceptible to PB and CPIB than normal hepatocytes. Histological examinations showed that PB and CPIB promoted

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* Abbreviations:

PB, phenobarbital;
CPIB, ethyl-α-*p*-chlorophenoxyisobutyrate;
DEN, diethylnitrosamine;
BD, basal diet;
HE, hematoxylin and eosin;
GSTP, placental form of glutathione S-transferase

preneoplastic lesions in the livers. The enhanced susceptibility is suggested to be responsible for the promotion of hepatocarcinogenesis.

Key words: Phalloidin sensitivity, Phenobarbital, Ethyl-α-p-chlorophenoxyisobutyrate, Rat preneoplastic hepatocytes

INTRODUCTION

Phenobarbital (PB*)(12, 13, 14, 28) and ethyl-α-p-chlorophenoxyisobutyrate (CPIB)(12, 16, 26) are well known as promoters of experimental hepatocarcinogenesis. Mechanisms of promotion have not been fully clarified as yet, although the acquisition of resistance of putative preneoplastic cells to cytotoxic agents(8, 9, 19) or mitogenic activity of the promoters(21) is suggested to be responsible for the promotion mechanisms.

Phalloidin is a hepatocyte-specific mushroom toxin. In *in vitro* experiments using isolated hepatocytes, multiple cytoplasmic blebs are formed over the cell surface by the toxin(29, 30). Decreases in the sensitivity to phalloidin were observed in hepatocytes isolated from rats treated with liver-tumor promoters(20, 27), putative preneoplastic hepatocytes(10, 18) and hepatoma cells of rats(6, 17, 18). The decreased sensitivity was suggested to depend primarily on the decrease in the inward transport of the toxin into the cells; thus, they depend on the changes in the cell membrane function(10, 17, 27).

In the present experiments, the effects of PB- or CPIB-feeding on the phalloidin sensitivity of preneoplastic hepatocytes are examined in order to clarify whether the cell membrane alterations are enhanced by the tumor promoters from the viewpoint that the enhancement is related to the promotion mechanisms.

MATERIALS AND METHODS

Animals.

Male SPF F344/DuCrj rats (Charles River Co., Atsugi) weighing 110-130 g were used. They were maintained in SPF animal facilities at 23±1℃ on a standard 12-hour light-dark daily cycle. They received diet according to the experimental schedules and drinking water *ad libitum*. They were acclimated to their environment for one week before the start of the experiments.

Experiment 1.

Experimental schedules are outlined in Fig. 1. Briefly, rats were fed a diet (MF, Oriental Yeast Co., Tokyo) containing 0.05% PB (Iwaki Seiyaku Co., Tokyo) or 0.25% CPIB (Shizuoka Caffeine Co., Shizuoka) for 14 weeks (group

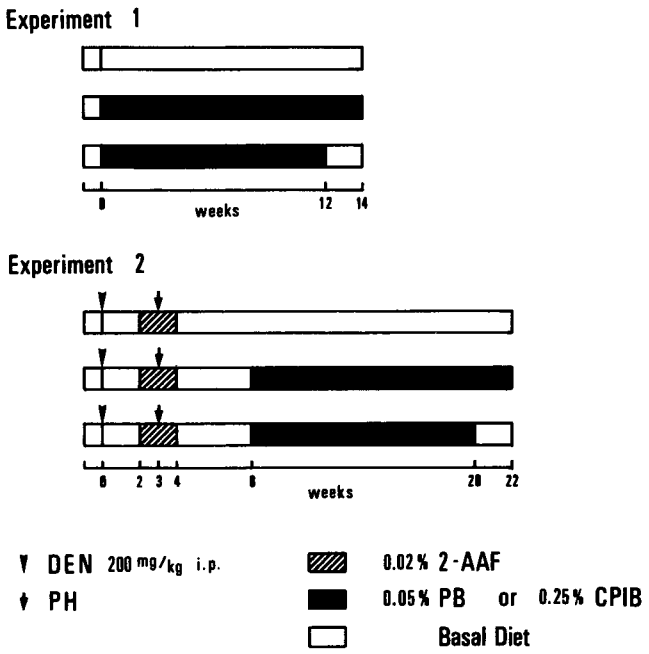

Fig. 1 Schematic representation of Experiments 1 and 2. PH, DEN, 2-AAF, PB and CPIB represent two-third partial hepatectomy, diethylnitrosamine, 2-acetylaminofluorene, phenobarbital and ethyl-α-p-chlorophenoxyisobutyrate, respectively.

PB or group CPIB) or 12 weeks. Rats treated with these compounds for 12 weeks were fed the basal diet (BD) for 2 successive weeks (groups PB+BD and CPIB+BD). Control rats were fed BD for 14 weeks (group BD).

Hepatocytes were isolated by a collagenase perfusion method as previously described(10). The isolated cells were finally suspended in Williams' Medium E (Flow Labs. Inc., Irvine, UK) supplemented with 10% calf serum (Hyclone Labs. Inc., Logan, USA), 0.5 μg/ml insulin, 0.04 mg/ml streptomycin sulfate and 40 IU per ml penicillin G. After the viability of the cells was determined by a trypan blue exclusion test (90-95% in these examinations), 3×10^5 viable cells in 1.5 ml of the medium were inoculated into 35-mm plastic dishes. After a one-hour cultivation in a CO_2-incubator at 37℃, the hepatocytes were incubated in 1.5 ml of the insulin-supplemented medium (without addition of calf serum) containing phalloidin (Sigma Chemical Co., St. Louis, USA) for 20 minutes at 37℃. The cells in the dishes were fixed in ice-cold ethanol for 10 min. The sensitivity of the cells to phalloidin was assessed as the percentage of the number of the cells that had formed multiple cytoplasmic blebs over the cell surface to the numbers of the observed cells.

Experiment 2.

Experiments basically followed the Solt-Farber protocol(24) as shown in Fig. 1. Briefly, rats were intraperitoneally injected with 200 mg/kg body weight diethylnitrosamine (DEN, Tokyo Kasei Indust., Tokyo) in 0.9% saline. After 2-week feeding on BD, they were fed a diet containing 0.02% 2-acetylaminofluorene (Tokyo Kasei) for 2 weeks. On the 21 st day after DEN injection, the rats received two-third partial hepatectomy. Thereafter, they were divided into 5 groups. Groups DEN+PB and DEN+PB+BD were fed a PB-diet for 14 weeks and 12 weeks, respectively, and groups DEN+CPIB and DEN+CPIB+BD were fed a CPIB-diet for 14 weeks and 12 weeks, respectively. Group DEN+BD received BD as a control. The animals were sacrificed following ether anesthesia at the 22 nd week after the DEN-injection.

After collagenase perfusion of the whole liver, only grayish or light brown nodules were carefully enucleated from the livers. The nodules were minced with scissors in the enzyme solution, and then teased with the aid of a 12 m*l*-injection syringe equipped with a 20-gauge needle. The specimens were further dissociated by bubbling with a gas mixture ($95\%O_2+5\%CO_2$) at 37℃ for 10 min. The isolated preneoplastic cells were finally suspended in Williams' Medium E supplemented with 10% calf serum, insulin and antibiotics. Phalloidin sensitivity was assessed as in Experiment 1.

Number and size of preneoplastic nodules in the liver tissues.

Rats following the same schedule as in Experiment 2 were used. After ether anesthesia, the rats were sacrificed, and then the livers were immediately removed. Two to three slices about 3 mm in thickness were cut from each lobe of the livers. They were fixed in ice-cold acetone and embedded in paraffin. Two adjacent sections were prepared, each of which was subjected to hematoxylin and eosin (HE)-staining and immunohistochemistry for the placental form of glutathione S-transferase (GSTP) using the avidin-biotion peroxidase method (Vectastain ABC Kit, Vector Labs. Inc., USA). The GSTP-stained preparations were photographed at a low magnification (×8), and the number of the GSTP-positive nodules was counted. The size of the nodules was measured on the photographs using an image analyzer (Kontron Co., Munich, FRG).

RESULTS

When either PB or CPIB was administered to normal rats for 14 weeks (group PB or group CPIB), the phalloidin sensitivity of the hepatocytes significantly decreased (Table 1). The sensitivity of the cells of rats in group PB and group CPIB were 73 to 90% and 88 to97% of the value of control (group BD), respectively,

depending on the concentration of phalloidin. After 2-week withdrawal of PB-feeding (group PB+BD) or CPIB-feeding (group CPIB+BD), the sensitivity of the hepatocytes recovered completely to the value of the control.

As shown in Table 2, preneoplastic hepatocytes isolated from the enucleated nodules in group DEN+BD were far more resistant to phalloidin than normal hepatocytes. The sensitivity of the cells in group DEN+PB decreased dramatically

Table 1 *Experiment 1: phalloidin sensitivity of hepatocytes isolated from rats treated with phenobarbital (PB) or ethyl-α-p-chlorophenoxyisobutyrate (CPIB)*

Treatment (group)	Number of rats	Phalloidin sensitivity		
		5[a]	10	20
Basal diet (BD)	6	87.0±2.6[b](100)[c]	92.6±2.1 (100)	94.7±1.8 (100)
PB	3	63.8±4.9[f](73.3)	79.0±0.4[f](85.3)	85.0±1.3[f](89.9)
PB+BD	3	85.8±1.3 (98.6)	90.7±0.6 (98.0)	92.0±1.3 (97.2)
CPIB	4	76.6±2.7[f](88.0)	88.4±1.6[d](95.5)	91.3±0.5[e](96.5)
CPIB+BD	4	85.6±1.2 (98.4)	91.5±1.1 (98.7)	93.1±0.7 (98.4)

[a] Concentration of phalloidin; μg/m*l*.

[b] Means±SD.

[c] Numbers in parentheses represent the percentage of each value compared to basal diet group.

[d,e,f] Significantly different from basal diet group: *d*, $P<0.05$; *e*, $P<0.01$; *f*, $P<0.001$.

Table 2 *Experiment 2: phalloidin sensitivity of preneoplastic hepatocytes isolated from rats treated with phenobarbital (PB) and ethyl-α-p-chlorophenoxyisobutyrate (CPIB)*

Treatment (group)	Number of rats	Phalloidin sensitivity		
		5[a]	10	20
Diethylnitros amine (DEN)+Basal diet (BD)	6	31.5±1.4[b](100)[c]	46.9±2.3 (100)	58.2±3.9 (100)
DEN+PB	5	8.8±0.9[f](27.9)	22.3±4.9[f](47.6)	31.6±6.5[f](54.2)
DEN+PB+BD	5	21.6±4.7[e](68.8)	35.4±6.4[e](75.5)	47.7±5.5[e](82.0)
DEN+CPIB	5	17.1±7.3[d](54.3)	26.6±8.6[e](56.7)	34.6±7.7[f](59.4)
DEN+CPIB+BD	4	30.2±2.9 (95.8)	40.7±1.9[e](86.8)	52.4±2.4 (89.9)

[a] Concentration of phalloidin; μg/m*l*.

[b] Means±SD.

[c] Numbers in parentheses represent the percentage of each value compared to DEN group.

[d,e,f] Significantly different from DEN group: *d*, $P<0.05$; *e*, $P<0.01$; *f*, $P<0.001$.

0.001.

as compared to that of the cells in group DEN+BD, falling to 28 to 54% of that in the latter. In comparison with normal hepatocytes, the effect of PB was amplified, being expressed in the preneoplastic cells with regard to the phalloidin sensitivity. After the 2-week withdrawal of PB-feeding (group DEN+PB+BD), the phalloidin sensitivity of the preneoplastic cells was partially recovered, showing 69 to 82% of the sensitivity in group DEN+BD.

Similar results were found in the preneoplastic cells induced by DEN+CPIB. The phalloidin sensitivity in group DEN+CPIB decreased markedly as compared to that in group DEN+BD, and the sensitivity of in the former was 54 to 59% of that in the latter. As compared to normal hepatocytes, the preneoplastic cells were markedly susceptible to the effect of CPIB. After the 2-week withdrawal of CPIB-feeding (group DEN+CPIB+BD), the sensitivity of the preneoplastic cells was recovered to show 87 to 96% of that in group DEN+BD.

As shown in Table 3, the average size of the nodules in both group DEN+PB and group DEN+CPIB increased to about 165% and 141% over that in group DEN+BD, respectively. After 2-week withdrawal of the agents, the average size in both group DEN+PB+BD and group DEN+CPIB+BD decreased, especially in the former. The number of the nodules per unit section area showed no differences among all groups. However, the liver weight increased considerably in group DEN+PB, indicating that the total number of the nodules in the whole liver had remarkably increased in this group.

DISCUSSION

Concerning the mechanisms of the promotion of hepatocarcinogenesis, much

Table 3 *Number and size of preneoplastic nodules in the livers of rats treated with phenobarbital (PB) or ethyl-α-p-chlorophenoxyisobutyrate (CPIB)*

Treatment (group)	Number of rats	Number of nodules (per cm²)	Average size of nodules (mm²)
Diethylnitrosamine (DEN)+Basal diet (BD)	7	46.3±6.2[a]	0.765±0.272 (100)[b]
DEN+PB	5	44.6±1.9	1.262±0.078[c](165)
DEN+PB+BD	5	46.5±5.9	0.870±0.282 (114)
DEN+CPIB	4	44.4±6.3	1.079±0.269 (141)
DEN+CPIB+BD	4	44.5±6.4	1.037±0.275 (136)

[a] Means±SD.

[b] Numbers in parentheses represent percentage of each value compared to the value of DEN group.

c Significantly different from DEN group: $P<0.01$.

interest was focused on the effects of promoters on cell membranes. Williams *et al.* reported that tumor promoters inhibited the intercellular communication in liver cell cultures(32), and that PB affected the activity of several membrane-associated enzymes in mouse liver tumors(31) and in cultured liver cells(22). On the other hand, the changes of gap junction(24) and the loss of asialoglycoprotein receptor(1) were reported to be caused in the hepatocytes by PB. Therefore, epigenetic changes in the cells which secondarily stimulate the preneoplastic cell proliferation might be induced by tumor promoters.

Our previous experiments showed that the sensitivity to phalloidin of putative preneoplastic rat hepatocytes(10) and also of hepatocytes of rats treated with various kinds of liver tumor promoters(3) decreased in parallel with the decrease in the uptake of the toxin by the cells. In the present experiments, the decrease in the phalloidin sensitivity of the promoter-treated preneoplastic hepatocytes was found to exceed the simple sum of the decrease in the untreated preneoplastic cells and that in the promoter-treated hepatocytes. These results suggest that the preneoplastic cells are more susceptible to PB and CPIB than normal hepatocytes with regard to phalloidin sensitivity.

After withdrawal of the tumor promoters, it has been reported that the majority of the preneoplastic lesions stopped growing further and that many of the lesions even disappeared(2, 5, 7, 23). In contrast, some authors did not observe such regression of the lesions after cessation of the promoter feeding(34). These conflicting observations may be ascribed to a variety of experimental designs, such as the duration of the promoter feeding, preneoplastic stages promoted, markers to identify histochemically the lesions, or contaminations of carcinogens in the diet(2). In the present experiments, we observed that, as to the phalloidin sensitivity, the effects of PB and CPIB were reversible completely in normal hepatocytes, but not completely in the preneoplastic cells in parallel with the reversibility of the size of the preneoplastic lesions.

Since phalloidin is considered neither to be metabolized in hepatocytes(15) nor to bind cytosomal ligandins(32), the changes in phalloidin sensitivity are to reflect the changes in the cell membrane functions. It is suggested that a reversible modification of the cell membranes induced by the promoters, which takes place more markedly in the preneoplastic cells, is responsible for the promotion of the liver lesion.

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