

# Modulation of Epidermal Growth Factor Receptors in Rat Hepatocytes by Two Liver Tumor-promoting Regimens, a Choline-deficient and a Phenobarbital Diet<sup>1</sup>

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## ABSTRACT

The effect of two liver tumor-promoting regimens, a choline-deficient (CD) and a phenobarbital (.06% PB) diet, on the level of epidermal growth factor (EGF) receptor in rat hepatocytes was examined at 3, 10, and 28 days of feeding. Both diets produced a significant decrease in the number of cell surface receptors at 10 and 28 days of treatment. When PB was included in a CD diet, the decrease in the receptor number was evident even after 3 days feeding of the combined diet. Neither diet alone had any effect on the binding at that time. Along with the changes in the receptor number, the binding affinity of EGF to its receptor was also altered by these diets. Furthermore, PB and PB plus CD diets also decreased the EGF binding at the intracellular sites whereas CD diet showed no effects indicating that the decrease in surface binding of EGF by the promoter-treated hepatocytes was not due to rapid internalization of the receptors. The reduced level of hepatocyte surface EGF receptors represents the common property shared by two diverse types of the liver tumor promoters, and may thus be related to the tumor-promoting ability of these agents.

## INTRODUCTION

Although it has been well established that PB<sup>3</sup> and CD diets act as liver tumor-promoting regimens (1-4), the mechanism by which these agents exert tumor-promoting effects is at present unknown. A CD diet induces alterations in phospholipid metabolism and results in abnormalities of some of the cell membrane-associated functions (5, 6). Since alterations of membrane receptors for growth factors are thought to play an important role in tumor promotion of other organ systems (7-9), we have sought to examine the role of these receptors in liver tumor promotion. Recently we demonstrated that a CD diet altered both the number and affinity of insulin receptors of rat hepatocytes (10). Whether the membrane alterations induced by a CD diet are limited to insulin receptors or affect receptors for other peptide growth factors is not known. Therefore, in this study we have investigated (a) whether a CD diet alters EGF receptors, (b) whether this alteration is produced by a different liver tumor promoter, namely PB, and (c) whether the degree of the alteration correlates with the biological potency of tumor promotion. The results show that both a PB and a CD diet induce a time-dependent decrease in the number of hepatocyte EGF receptors and the combination of the two agents enhances this effect. This is the first demonstration of the common cellular responses by two diverse types of liver tumor-promoting regimens and the changes may be linked to the underlying mechanisms of liver tumor promotion.

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<sup>3</sup> The abbreviations used are: PB, phenobarbital; EGF, epidermal growth factor; CD, choline deficient; CS, choline supplemented; MEM, minimum essential medium.

## MATERIALS AND METHODS

**Animal and Diets.** Male Sprague-Dawley rats (Zivic-Miller Laboratories, Allison Park, PA) weighing 180 to 200 g were used. The animals were housed individually in metal cages in a room with temperature, humidity, and light controls and maintained on Purina chow (Ralston Purina, St. Louis, MO). Semisynthetic semipurified CS and CD diets were prepared as described previously (3) and PB (Sigma Chemical Co., St. Louis, MO) at a level of 0.06% was incorporated into batches of each diet at the expense of sucrose. All animals were fed standard laboratory chow and 7 days later they were placed on the experimental diets. The animals were divided into 5 groups and were fed the commercial chow, CS, CS + PB, CD, and CD + PB diets, respectively. Five to seven rats in each group were killed after 3, 10, and 28 days of feeding.

**Hepatocyte Isolation.** The separation of liver cells was accomplished using the two-step collagenase perfusion technique described by Seglen (11) and modified by Jirtle *et al.* (12). The method involves perfusion of the liver through portal vein first with 250 ml of buffer [142 mM NaCl, 6.7 mM KCl, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4] and then with 0.05% collagenase (Cooper Biomedical, CLS, II) in the above medium containing 5.7 mM CaCl<sub>2</sub>. After 15 min of perfusion, the liver capsule was opened with several cuts and the cells were dispersed in MEM. The cells were washed 3 times by centrifuging at 20 × g for 3 min, and a clean single cell suspension of hepatocytes was obtained. Viability of the hepatocytes was judged by trypan blue exclusion, and the preparations with a viability of >90% were used for further studies.

For a short-term culture of hepatocytes, a portion of the cells was placed in culture medium (MEM) containing 1 mM pyruvate, 0.2 mM aspartate, 0.2 mM serine, 40 μg/ml gentamycin, 10<sup>-7</sup> M insulin, and 5% fetal calf serum. After attachment of the cells on the plate (approximately 4 h), the medium was removed and fresh medium without fetal calf serum but containing 0.1% bovine serum albumin was added. The cells were incubated in this medium for 1 to 18 h before using in EGF binding assays.

**EGF Binding.** The receptor assay was carried out with freshly prepared cells as well as cultured hepatocytes placed on the culture medium for 4 h. The assay was based on the method by Carpenter (13). Briefly, 10<sup>6</sup> cells were incubated at 4°C with different concentrations of <sup>125</sup>I-EGF (0.2-2 nM) in the presence and absence of unlabeled EGF (1 μg/ml; Sigma) for 1 h in MEM containing 0.1% bovine serum albumin. <sup>125</sup>I-EGF (138 mCi/μg) was purchased from NEN, Boston, MA. At this time the binding by 10<sup>6</sup> cells reached a plateau. The cells were then centrifuged at 500 × g, washed twice with the above medium to remove unbound radioactivity, and the bound radioactivity was measured using a gamma counter. The specific binding was determined by subtracting the nonspecific binding per 10<sup>6</sup> cells from the total binding per 10<sup>6</sup> cells. The number of binding sites and dissociation constant were obtained from computer-assisted nonlinear weighted least-squared regression analysis of the data using computer program LIGAND (G. A. McPherson, Elsevier-Biosoft, Elsevier Science Publisher, Cambridge, 1985).

Other sets of experiments were carried out in which incubations were performed at 37°C. After incubation of cells in the assay medium at 37°C for 1 h, the cells were washed with 0.2 M acetate buffer, pH 3.0. This procedure washes the binding due to cell surface receptor, but the internalized binding activity remains unaffected (13). Thus, using this procedure, we are able to estimate the net intracellular binding activity.

**Data Analysis.** The results were evaluated by two-way analysis of variance.

## RESULTS

**EGF Binding at 4°C.** The results in Table 1 show the effect of chow, CS, CD, CS + PB, and CD + PB diets on EGF binding to hepatocytes at 4°C which represent the receptor number on the surface of hepatocytes. Although most of the data represent the binding experiments with freshly isolated cells, some experiments (PB- and CS-fed animals) were repeated using cultured cells (4–18 h) and no difference in the binding data was noted among fresh or cultured cells. Thus, it appears that the collagenase did not interfere with the EGF binding assays on freshly isolated hepatocytes. Although a CS diet is the control diet for PB and CD diets and is nutritionally adequate to sustain normal growth of rats, the hepatocyte EGF receptor number in CS-fed rats was found to be lower than that in chow-fed rats. This effect is most likely due to the compositional differences of the diets between commercial chow and a semisynthetic CS diet.

Since CS diet is the appropriate control diet for other experimental treatments, the results were compared with that in CS-fed animals. The CD and PB diets produced no significant effect following 3 days on the diets. After 10 days feeding, CD and PB diets induced 53 and 60% decreases, respectively, of the surface receptor number. While PB induced no further reduction in receptor number following 28 days of feeding, a CD diet showed a slight recovery (Fig. 1) at that time. The combination of a CD and a PB diet produced a greater decrease in the receptor number than that observed with either a PB or a CD diet alone (Table 1; Fig. 1). A significant decrease was evident even at 3 days of feeding when neither of the regimens alone had any effect on the binding. The decreases remained after 10 and 28 days of feeding. Thus, the combination of PB and CD diets not only accentuated the decrease in EGF binding by PB or CD diets but also accelerated their response.

**Dissociation Constant.** The effect of the experimental diets on the dissociation constants of EGF receptor determined at 4°C is shown in Table 2. Saturation plots and Scatchard analysis of different binding curves on the effect of various diets are shown in Fig. 2. Nonspecific binding constituted 8–10% of the total binding. The linear nature of these binding curves indicates that rat hepatocytes have a single class of EGF receptors which are affected by various promoters, causing changes in the dissociation constant. All of these diets, either alone or in the combination, produced a decrease (50%) in the dissociation constant at 10 and 28 days of feeding. At 3 days, only PB and CD + PB diets produced a decrease in the dissociation constant, whereas a CD diet alone had no effect on it. Thus, at Day 3 a PB diet affected the dissociation constant of the receptor binding to EGF, even though it had no effect on the receptor number.

**EGF Binding at 37°C.** In order to determine the intracellular

binding activity, some of the binding experiments were carried out at 37°C. The intracellular binding activity of hepatocytes from rats treated with different promoting regimens is shown in Table 3. The amount of EGF-bound receptor present in the intracellular sites of hepatocytes from PB-treated rats was significantly lower than that of hepatocytes from CS-treated rats. The results correlate with the surface binding activity (Table 1), i.e., both the cell surface and intracellular binding of PB-fed hepatocytes were not altered at 3 days but were decreased at later time points. The intracellular binding of CD diet-fed hepatocytes was not different from that of the control at any of the time points. In hepatocytes of rats fed a CD + PB diet, however, the intracellular binding was lower than that in hepatocytes of rats fed a CS diet.

## DISCUSSION

The results presented here show that two prototypes of the liver tumor-promoting regimens, a PB and a CD diet, altered the EGF receptor binding activity. Not only the number of cell surface receptors but also their affinity to the ligand was changed. When these two diets were combined, i.e., PB + CD diet, the decrease in receptor number was accentuated over that produced by either of the diets alone. This augmenting effect was particularly evident in the rats following 3 days of feeding the diets, and the effect was less evident at later time points. Earlier, we showed that the combination of a PB and a CD diet exerted a stronger promoting action as assayed by the induction of enzyme-altered foci in the carcinogen-initiated rats than each agent alone (14). It is thus reasonable to suggest that the overall changes of the hepatocyte EGF receptors may be in part related to the promoting action of the two agents. It has been shown that the number of EGF receptors decreases during rat liver regeneration (15, 16). The present findings, however, negate the possibility that the decrease in EGF receptor number in the liver cells of rats fed a CD diet or a CD + PB diet is related to the CD diet-induced liver cell proliferation since PB, which inhibits CD diet-induced liver cell proliferation (17), also produced this effect.

Even though a PB and a CD diet exert the common property of liver tumor promotion, they often differ in their biological and biochemical effects on the liver. PB is a well-known inducer of microsomal mixed function oxidase and the induction of these enzymes was suppressed by a CD diet (18, 19). A CD diet also induces liver cell proliferation whereas feeding of PB suppresses it (17). A CD diet induces hepatocyte membrane lipid peroxidation, but PB has no such effect (20, 21). As far as we are aware, the cell surface receptor alterations reported here represent the first biochemical effect shared by these agents. The mechanism by which tumor promoters decrease cell surface binding is unclear. If this was due to higher internalization of the surface receptor under *in vivo* conditions, one would expect a higher number of EGF receptors at the intracellular sites.

Table 1 Effect of CS, CD, CS + PB, and CD + PB diets on cell surface EGF receptor

Treatment	EGF binding (fmol/10 <sup>6</sup> cells) <sup>a</sup>		
	3 days	10 days	28 days
Chow	168.3 ± 10.2	168.3 ± 10.2	168 ± 10.2
Choline-sufficient	126.7 ± 11.6	117.6 ± 76.5	97.3 ± 14.8
Choline-deficient	109.5 ± 21.7 (13%) <sup>b</sup>	57.5 ± 6.7 (51%)	72.4 ± 7.2 (36%)
Choline-sufficient plus phenobarbital	112.3 ± 8.2 (12%)	40.1 ± 8.7 (66%)	38.7 ± 7.2 (60%)
Choline-deficient plus phenobarbital	56.6 ± 23.2 (55%)	25.6 ± 6.6 (78%)	24.7 ± 8.7 (75%)

<sup>a</sup> Significant differences ( $P < 0.001$ ) exist between control group (CS) and treatment groups as well as between choline-deficient plus phenobarbital and other treatment groups by analysis of variance (two-way analysis) test. Data represent mean ± SD,  $n = 4-7$ .

<sup>b</sup> No. in parentheses indicates % of decrease from CS value.

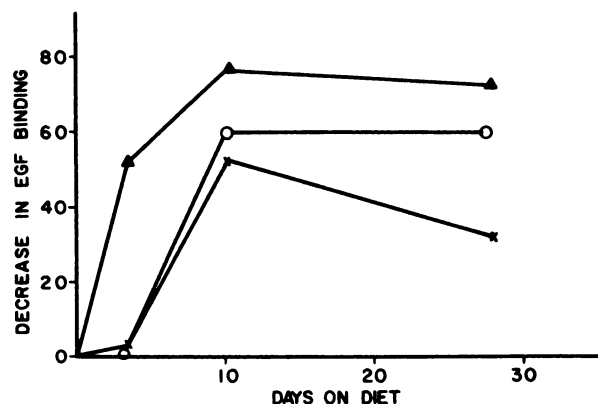


Fig. 1. Decrease in surface EGF receptor in the hepatocytes of rats fed CD (x), PB (o), and PB + CD (Δ) diets for different time periods. The data represent a mean of 4-6 determinations using different rats.

Table 2 Effect of CS, CD, CS + PB, and CD + PB diets on dissociation constant of EGF receptors

Treatment	Dissociation constant of EGF receptor (nM) <sup>a</sup>		
	3 days	10 days	28 days
Chow	1.01 ± 0.12	1.12 ± 0.10	0.97 ± 0.15
Choline-sufficient	0.87 ± 0.18	0.81 ± 0.11	0.80 ± 0.22
Choline-deficient	0.85 ± 0.21	0.52 ± 0.13	0.47 ± 0.26
Choline-sufficient plus phenobarbital	0.46 ± 0.13	0.44 ± 0.09	0.47 ± 0.08
Choline-deficient plus phenobarbital	0.49 ± 0.14	0.43 ± 0.11	0.40 ± 0.12

<sup>a</sup> Significant differences ( $P < 0.001$ ) exist between control (CS) and treatment groups by analysis of variance test. Data represent mean ± SD.

The measurements at 37°C, however, do not support this possibility, as we found either a decreased or unaltered concentration of this protein at the intracellular sites in the promoter-treated liver hepatocytes. On the other hand, a change in the rate of degradation of EGF receptor at the intracellular sites may be another factor that could be involved in the alteration of EGF binding at the intracellular sites. The significance of

the difference in the intracellular bindings of EGF between a PB and a CD diet is not clear at the present time.

Alterations of growth factor receptors by liver tumor promoters have been indicated in various other studies (10, 22-24). Jirtle *et al.* and Eckl *et al.* reported a decrease in surface EGF receptor concentration in hepatocytes of rats fed PB for 4 wk (22, 23). Changes in the EGF and insulin binding to the golgi and microsomal fractions of the liver of rats fed phenobarbital were also demonstrated by Hwang *et al.* (24). They showed that phenobarbital treatment decreased the number of EGF receptors but did not alter their affinity. The present study shows the changes not only in the concentration of EGF receptor but also in their binding affinity by these agents. Whether these promoters alter certain cell surface receptors selectively is yet to be determined. Evarts *et al.* reported an alteration of surface asialoglycoprotein receptor by PB (25). Our preliminary studies also indicate that PB modifies hepatocyte insulin receptors similar to the CD diet, but it has no effect on glucagon receptors (26). Possible involvement of cell surface receptor alterations in the process of liver tumor induction is also suggested by Harris *et al.*, who demonstrated the progressive decreases of EGF bindings in normal hepatocytes, preneoplastic hepatocytes, and hepatoma cells during hepatocarcinogenesis (27). Alterations of phospholipid composition of cell membranes can lead to various biochemical and biological changes in the membrane (28, 29). PB is known to induce changes in the phospholipid composition of cell membranes (30, 31), and a CD diet alters phospholipid metabolism (32). Thus, they may induce changes in the phospholipid composition of cell membrane and may result in an alteration of cell surface receptors. Alternatively, the receptor changes induced by a CD diet may be the results of peroxidative damage of cell membrane lipids (20).

Control of liver cell growth and proliferation is complex, and a number of peptides such as growth hormones, EGF, insulin-like growth factors, and glucagon have been implicated to participate in the process (33). Furthermore, several liver cell-

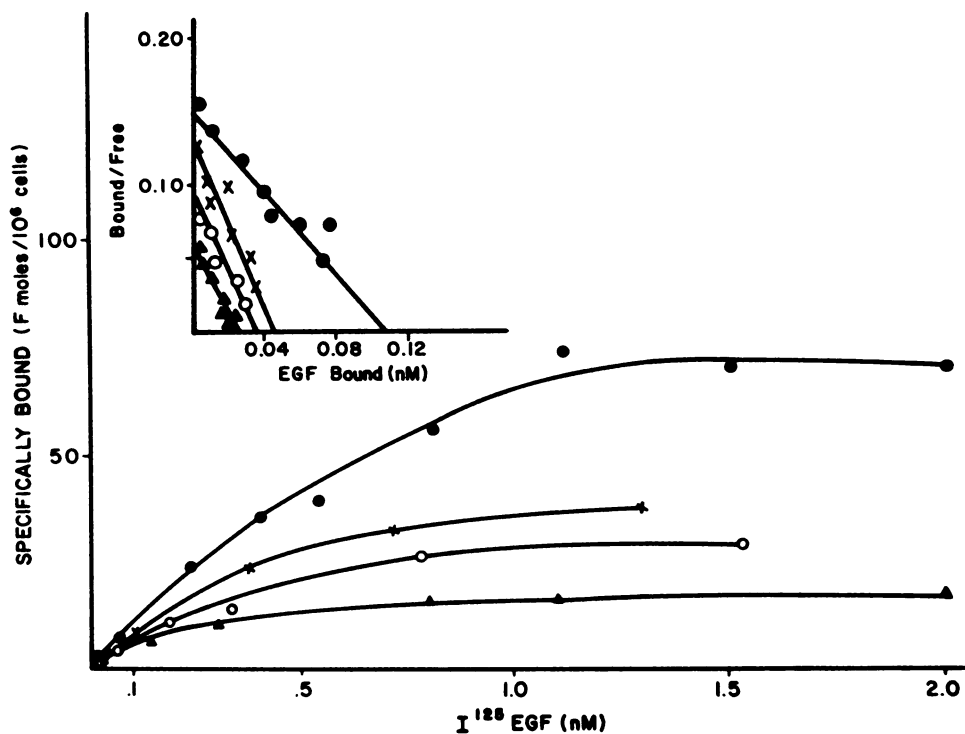


Fig. 2. Saturation plot of EGF binding at 4°C by hepatocytes from CS (●), CD (x), PB (○), and PB + CD (Δ)-fed (10 days) rats. 10<sup>6</sup> cells/ml were used in each determination. The data represent a mean of 4-6 determinations using different rats. Inset, Scatchard analysis of the binding data.

Table 3 Effect of CD, CS, CS + PB, and CD + PB diets on intracellular binding of EGF

Treatment	fmol/10 <sup>6</sup> cells <sup>a</sup>		
	3 days	10 days	28 days
Choline-sufficient	178 ± 42.2	167.5 ± 42.7	151.2 ± 24.6
Choline-deficient	155 ± 31.8 (13%) <sup>b</sup>	143.7 ± 24.9 (15%)	156.0 ± 29.3 (9%)
Choline-sufficient plus phenobarbital	130 ± 34.0 (27%)	68.5 ± 7.5 (59%)	71.2 ± 19.1 (53%)
Choline-deficient plus phenobarbital	66.2 ± 11.7 (63%)	54.2 ± 16.5 (68%)	67.0 ± 21.2 (56%)

<sup>a</sup> Choline-sufficient plus phenobarbital and choline-deficient plus phenobarbital groups are significantly different from choline-sufficient group by analysis of variance test (two way analysis). Data represent mean ± SD.

<sup>b</sup> No. in parenthesis indicates % of decrease from CS value.

specific growth factors have been reported (34–37), which may also exert their effects through receptor-mediated events. More recently, transforming growth factor type  $\beta$  has been shown to be involved in the control of liver cell proliferation (38, 39). It is conceivable that changes in any one of the cell surface receptors involved in liver cell growth may lead to disturbance of hemostatic growth control of the liver cells, and such changes may play a critical role in liver tumor promotion.

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