Effects of the Liver Tumor Promoter Ethinyl Estradiol on Epidermal Growth Factor-induced DNA Synthesis and Epidermal Growth Factor Receptor Levels in Cultured Rat Hepatocytes

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ABSTRACT

The objective of this study was to determine whether DNA synthesis induced in the livers of female rats treated with ethinyl estradiol (EE) was due to direct effects of this synthetic estrogen on hepatocytes. Hepatocytes, obtained by collagenase perfusion from female Lewis rats, were cultured in serum-free medium containing low or no phenol red and supplemented with insulin, transferrin, and selenium. When present at 10–15 μM for the initial 30 h of culture, EE caused a subsequent 2–2.7-fold increase in hepatocyte DNA synthesis. Pretreatment of the hepatocytes with EE during the first 30 h of culture caused an EE concentration-dependent enhancement of their subsequent DNA synthetic response to epidermal growth factor (EGF). Pretreatment with EE shifted the EGF dose-response curve, causing a dramatic enhancement of the response to EGF beginning at 2 ng EGF/ml. The response to a saturating (25 ng/ml) dose of EGF was also greatly enhanced. Determination of the effect of EE on hepatocyte surface EGF receptors revealed that the increased responsiveness of DNA synthesis to EGF was accompanied by a twofold increase in EGF receptor number per cell. These results indicate that EE has direct, growth-related effects on hepatocytes which may contribute to liver growth induced in vivo by this tumor promoter.

INTRODUCTION

Results from our laboratory (1, 2) and several others (3–5) have established that certain synthetic estrogens such as mestranol and EE are strong promoters of hepatocarcinogenesis in rats and mice. These agents may also have some weak complete carcinogenic activity (6, 7) although it is possible that such activity may actually represent promotion of spontaneously initiated hepatocytes (8) since the synthetic estrogens lack detectable ability to initiate hepatocarcinogenesis (9, 10).

The ability to cause increased cell proliferation is clearly an important property of tumor promoters (11). Schulte-Hermann et al. (12) have demonstrated that cellular proliferation is preferentially enhanced in preneoplastic versus normal liver tissue by several liver tumor promoters. Previously, we demonstrated that low doses of mestranol and EE rapidly stimulated liver DNA synthesis in a dose-dependent manner (13). Upon continuous treatment with these promoters, elevated levels of hepatocyte DNA synthesis persisted for at least 7 days before returning to basal levels by 14 days. Furthermore, we found that these dose levels did not cause a loss of [3H]thymidine from prelabeled liver DNA indicating that the enhanced DNA synthesis was not a result of regenerative hyperplasia in response to hepatotoxicity of the synthetic estrogens. However, neither our studies nor those of others provided information pertaining to whether the hyperplastic response induced by EE occurred through direct or indirect effects on hepatocytes.

Several reports have appeared demonstrating that various liver tumor promoters stimulate DNA synthesis in adult rat hepatocytes in primary culture. Kitagawa and coworkers cultured hepatocytes from rats initiated by carcinogen treatment (14). They found that PB, but not TPA, enhanced the growth of foci of growing epithelial cells. Miyazaki et al. (15) reported prolonged survival of adult rat hepatocytes in primary cultures treated continuously with 3 mM PB, although PB did not appear to stimulate hepatocyte growth. On the other hand, Edwards and Lucas (16) have shown that several liver tumor promoters, including PB at 3 mM, caused a concentration-dependent stimulation in hepatocyte DNA synthesis. These differences may have been caused by use of different media, hormonal supplements, and culture conditions. Recently, Sawada et al. (17) reported that in serum-free medium supplemented with insulin, selenium, transferrin, and dexamethasone, both TPA and PB enhanced the adult rat hepatocyte DNA synthetic response induced by EGF.

These studies establish the precedent for use of adult rat hepatocytes in primary culture in order to study the effects of liver tumor promoters on hepatocyte DNA synthesis. The goal of the present study was to investigate whether EE has direct effects on hepatocytes which lead to stimulation of DNA synthesis. This report describes the results of our studies on the effects of EE on DNA synthesis in female rat hepatocytes in culture in the presence and absence of EGF. EE was found to dramatically potentiate the DNA synthetic response of hepatocytes to EGF and to increase the number of EGF receptors per cell.

MATERIALS AND METHODS

Animals. Female Lewis rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were housed under controlled conditions of temperature, humidity, and light and received food and water ad libitum as described previously (13). The dark period ended at 10:00 a.m. and all perfusions were performed between 8:00 a.m. and 10:00 a.m.

Special Materials. Collagenase type I was obtained from Cooper Biomedical, Malvern, PA. The media, Ham's F-12 and DMEM (phenol red free), were purchased from GIBCO Laboratories, Grand Island, NY. The insulin-transferrin-selenium preparation and EGF were from Collaborative Research Inc., Bedford, MA. Ethinyl estradiol and 17β-estradiol were purchased from Sigma Chemical Company, St. Louis, MO. [3H]Thymidine and the [3H]estradiol binding assay kit were from New England Nuclear Corp., Boston, MA. In most experiments [6,3H]thymidine was used, however, in a few experiments [methyl-3H]thymidine was used.

Hepatocyte Isolation and Culture. Hepatocytes were isolated from female Lewis rats weighing between 130 and 180 g using a modification of the two-step collagenase perfusion technique of Seglen (18) as described previously (19). The isolated hepatocytes were inoculated into collagen coated (20) culture dishes at a density of 2.8 x 10⁴ cells/cm² in our basal serum-free medium which consisted of a 1:1 mixture of Ham's F-12 and DMEM supplemented with insulin-transferrin-sele-
nium (insulin, 5 µg/ml; transferrin, 5 µg/ml; selenium, 5 ng/ml). The cultures were maintained at 37°C in a 5% CO₂ atmosphere. Phenol red has been shown to have weak estrogenic activity (21). In the present study, the final concentration of phenol red in the medium was either 0.6 µg/ml (due to the phenol red in Ham’s F-12) or it was completely absent when both media were obtained phenol red free. (Standard DMEM contains phenol red at 15 µg/ml). However, since we found that the presence of phenol red did not alter the effects of EE we were measuring (data not shown), we have not distinguished between experiments where it was present or absent.

The protocol used in the majority of the experiments being reported is shown in Fig. 1. Following inoculation of the cells in basal medium, an initial 4-h period was allowed for attachment and then the medium was changed (0 time) to remove unattached cells. The hepatocytes were next exposed to EE or other agents from 0 to 30 h ± EGF from 18 to 30 h whereupon the hormones were removed by a change to basal medium. However, in several experiments EGF was added at time 0.

EE was dissolved in 70% ethanol and then diluted to 35% ethanol with medium. After addition to the cultures, the final ethanol concentration was 0.17%. Controls were exposed to the same concentration of ethanol.

[^3H]Thymidine Incorporation. The cells were exposed to[^3H]thymidine (3–4 µCi/culture) for various periods at different times prior to harvest (Fig. 1). Radioactivity in DNA extracted with hot trichloroacetic acid (13) was determined using a Beckman 7000 liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). The incorporation of[^3H]thymidine into DNA was expressed as dpm/mg cellular protein.

Protein content was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as a standard.

Autoradiography. In experiments assayed by autoradiography,[^3H]thymidine was added at a concentration of 3 µCi/culture. At the time of harvest, the cells were washed five times with phosphate buffered saline, fixed with 10% formalin, covered with Kodak Nuclear Emulsion (Eastman Kodak, Rochester, NY), diluted 1:1 with water, and stored in the dark for 10 days. At the end of the exposure period, the coated plates were developed with Kodak Developer D-19 and fixed with Kodak Fixer. The plates were stained with hematoxylin & eosin. The labeling indices were determined from counts of 300–400 nuclei and expressed as the percentage of nuclei counted that were heavily labeled.

Analysis of Estrogen Receptor Levels. Analysis of[^3H]estradiol binding on cytosols prepared from rat liver and isolated hepatocytes was conducted according to the instructions supplied with the estrogen receptor assay kit from New England Nuclear. The binding data were analyzed according to the method of Scatchard (22).

Fig. 1. Protocol for experiments to determine the effects of EE pretreatment on hepatocyte DNA synthetic response to EGF.

**RESULTS**

Stimulation of Hepatocyte DNA Synthesis by EGF. Hepatocyte DNA synthesis in response to continuous exposure (0–48 h) to EGF was determined using 2-h pulses of[^3H]thymidine and is shown in Fig. 2A. After an initial lag of about 24 h, DNA synthesis increased and reached a peak at 48 h. Little change in DNA synthesis was observed in the absence of EGF. The inset in Fig. 2A shows that at 48 h, the maximal DNA synthetic response was observed at 25 ng EGF/ml (4.1 nM). Determination of the nuclear labeling index showed that the increased[^3H]thymidine incorporation into DNA which occurred in response to EGF reflects an increase in the number of hepatocytes undergoing DNA synthesis (see below). The levels of acid soluble radioactivity were not affected by EGF or the other agents used in this study (data not shown). Furthermore, the changes in[^3H]thymidine incorporation were inhibited by hydroxyurea (data not shown). Sawada et al. (17) found that neither EGF, PB, or TPA altered the specific activity of[^3H]thymidine pools in cultured hepatocytes. Taken together, these results indicate that the increased[^3H]thymidine incorporation into DNA reflects replicative DNA synthesis and is not due to treatment-induced alterations in isotope uptake.

Next, we determined the effects of the time of addition and length of exposure to EGF on hepatocyte DNA synthesis. In an initial experiment, cultures were exposed to EGF continuously or for various shorter periods of time. DNA synthesis was determined using a pulse of[^3H]thymidine from 46 to 48 h. The results (data not shown) indicated that EGF must be present continuously (0–48 h) in order to attain the maximal response. However, a significant response was observed when EGF treatment was limited to the 12-h period between 18 and 30 h of culture. Figure 2B shows the results of an experiment comparing the time course of DNA synthesis in cultures exposed to 25 ng/ml EGF continuously or only between 18 and 30 h. In the latter cultures, EGF was added at 18 h and at 30 h the medium was changed to basal medium (i.e., the EGF was removed). The cultures were exposed to[^3H]thymidine for 2 h at the times indicated. The data show that DNA synthesis increased more rapidly and overall achieved higher levels with continuous exposure. However, under both conditions of exposure the response pattern was similar with peaks at 48 h.

Effect of EE on Hepatocyte DNA Synthesis. We employed this culture system to determine whether EE has direct effects on hepatocyte DNA synthesis. Initially, we determined the levels of cytosolic estrogen receptors by analysis of specific[^3H]estradiol binding in unfractionated cytosol prepared from intact rat liver, collagenase perfused liver, and isolated hepatocytes. The data (not shown) from binding experiments resulted in linear Scatchard plots which showed high affinity binding, K_d = 4 × 10^{−10} M. The binding capacity of intact liver was 31 fmol/mg protein, a value within the range of that seen by others.
significant interaction between EE and EGF, P = 0.034. In another experiment, hepatocyte cultures were exposed to EE (15 nM), EGF (25 ng/ml), or EE plus EGF from 0 to 30 h; ['H]thymidine was present from 30 to 48 h, i.e., during the ascending portion of the EGF response curve (Fig. 2). The results (data not shown) showed a concentration-dependent increase in DNA synthesis which was 1.7-, 1.9-, 2.2-, 2.6-, and 2.7-fold over controls at 2.5, 5, 10, 15, and 20 nM EE, respectively.

In another experiment, hepatocyte cultures were exposed to EE (15 nM), EGF (25 ng/ml), or EE plus EGF from 0 to 30 h; ['H]thymidine was present from 30 to 48 h, i.e., during the ascending portion of the EGF response curve (Fig. 2). The results, Table 1, show that EE alone caused a 2.2-fold increase over control in DNA synthesis as compared to EGF alone which stimulated DNA synthesis 10.7-fold over control. However, in cultures exposed to EE and EGF together, DNA synthesis was stimulated 32-fold. Statistical analysis of this data indicated a significant interaction between EE and EGF, P = 0.034. Thus, the response in the presence of both hormones together was significantly enhanced compared to that seen with either hormone alone.

Table 1  Effect of EE on the stimulation of hepatocyte DNA synthesis by EGF

<table>
<thead>
<tr>
<th>Treatment</th>
<th>dpm x 10^6/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>131 ± 10^6</td>
</tr>
<tr>
<td>EE</td>
<td>291 ± 15</td>
</tr>
<tr>
<td>EGF</td>
<td>1404 ± 40</td>
</tr>
<tr>
<td>EE + EGF</td>
<td>4196 ± 737</td>
</tr>
</tbody>
</table>

The cultures were exposed to EE (15 nM) and/or EGF (25 ng/ml) for 0-30 h and to ['H]thymidine for 30-48 h. Hepatocyte DNA synthesis was determined as described in "Materials and Methods."

Mean ± SD of triplicate cultures; analysis of variance indicated that all treated groups were significantly greater than control and that there was a significant interaction between EE and EGF, P = 0.034.
Fig. 3. The time course of DNA synthesis in hepatocytes exposed to EE, EGF, or EE followed by EGF. At 0 time, cultures were exposed to vehicle, (C, control), or 15 μM EE alone (EE), 18 h later, EGF (25 ng/ml) was added to non-EE pretreated (□) or EE pretreated (■) cultures. 12 h later, the media were changed to basal medium, thus removing the hormones (see Fig. 1). The cultures were harvested at the times indicated following a 2-h pulse with \[^{3}H\]thymidine. Each point represents the mean ± SD of triplicate cultures.

Table 2 \[^{3}H\]Thymidine nuclear labeling index in hepatocytes exposed to EE, EGF, and EE + EGF

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.3 ± 0.5</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>EE</td>
<td>4.1 ± 0.7</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>EGF</td>
<td>7.2 ± 1.2</td>
<td>4.3 ± 0.6</td>
</tr>
<tr>
<td>EE + EGF</td>
<td>20.8 ± 2.5</td>
<td>14.6 ± 0.6</td>
</tr>
</tbody>
</table>

* The cultures were exposed to 15 μM EE (0–30 h), 25 ng/ml EGF (18–30 h), or both EE (0–30 h) + EGF (18–30 h) followed by \[^{3}H\]thymidine from 30 to 48 h. These data are from two separate experiments.

** Mean ± SD of triplicate cultures based on counting 300 cells per culture. 

The effect of the presence of various concentrations of EE on the responsiveness of hepatocyte DNA synthesis to EGF is shown in Table 3. The cells were exposed to EE (2.5–20 μM) from 0 to 30 h and EGF (25 ng/ml) from 18 to 30 h; \[^{3}H\]thymidine was present from 30 to 48 h. The results show an EE dose-dependent enhancement of EGF-induced DNA synthesis. An analysis of variance indicated a significant interaction between EGF and EE at all EE concentrations. For example, for the control, EE (15 μM), EGF, and EE (15 μM) + EGF groups, the test of interaction yielded P ≤ 0.0001. With EGF plus EE at 10 and 15 μM, the increase in DNA synthesis over control was 13.1- and 13.9-fold, respectively, compared to a 3.1-fold increase caused by EGF alone and a 2.6-fold increase caused by 15 μM EE alone. The concentrations of EE being used are relatively high. However, the results of a study, to be reported elsewhere, demonstrated that greater than 90% of the EE present in the cultures was metabolized to conjugated derivatives within 4 h of addition. This may account for the requirement for μM concentrations of EE in these studies.

Next, an experiment was conducted to determine the effects of EE pretreatment on the response of hepatocyte DNA synthesis to EGF concentration. In this experiment, EE (15 μM) was present from 0 to 30 h and EGF, at the concentration indicated, from 18 to 30 h; \[^{3}H\]thymidine was present from 30 to 48 h. As shown in Fig. 4, in the absence of EGF, the stimulatory effect of EE was 2.4-fold. Upon pretreatment of the hepatocytes with EE, the EGF dose-response curve was shifted to the left and the response observed at saturating concentrations of EGF was dramatically increased. Thus, 2 ng/
ml EGF alone stimulated DNA synthesis 1.6-fold whereas with EE pretreatment the increase over control was 6.2-fold. At 25 ng/ml EGF alone the increase over control was 5-fold whereas with EE pretreatment the effects were than those observed with EE (data not shown).

Effect of EE on Hepatocyte EGF Receptor Levels. The results presented above indicate that EE pretreatment increased the responsiveness of cultured hepatocytes to EGF. We hypothesized that perhaps this effect was associated with an alteration of the hepatocyte surface EGF receptor levels. Fig. 5 shows a representative Scatchard plot of the effect of EE on the equilibrium binding of 125I-EGF to intact cultured hepatocytes. Table 4 shows data from three independent experiments (cells from three different rats). In these experiments the hepatocytes were exposed to EE (2 μM) for 18 h at which time the equilibrium binding of 125I-EGF was determined as described in "Materials and Methods." In a typical experiment, this would be the time of addition of EGF although in these experiments, EGF was not added. The results show that EE caused a significant (P < 0.001) twofold increase in EGF receptor number per cell compared to control cultures. There was also a small but significant (P < 0.02) 22% decrease in the $K_d$. In a subsequent experiment, the maximum stimulation of 125I-EGF binding (2.3-fold) was observed at 5–10 μM EE. Thus, the dramatic potentiation of EGF-induced hepatocyte DNA synthesis, caused by pretreatment with EE, is associated with a twofold increase in surface EGF receptor number and a small increase in ligand binding affinity.

DISCUSSION

In previous studies, we (13) and Ochs et al. (27) demonstrated that the liver tumor promoter EE stimulated a transient increase in rat hepatocyte DNA synthesis. However, it was not known whether growth stimulation by EE occurred through direct or indirect effects on hepatocytes. The goal of the present study was to determine whether EE has direct, growth-related effects on rat hepatocytes. Our data demonstrate that EE alone caused a 2–2.7-fold stimulation of DNA synthesis in cultured hepatocytes. However, pretreatment with EE dramatically potentiated their DNA synthetic response to EGF. Furthermore, this effect was associated with a twofold increase in cell surface EGF receptor number and a small but statistically significant decrease in $K_d$.

Our results with hepatocytes in primary culture represent an example of heterologous regulation of the EGF receptor, a phenomenon observed by others in several different cell systems (28–30). Perhaps the best known example of heterologous regulation is mediated by the tumor promoter TPA which is a potent activator of protein kinase C (31). Treatment of cells with TPA generally causes a down-regulation of the EGF receptor.

There are examples, however, where hormone treatment has resulted in an increase in EGF binding. In vivo in mice, estrogen stimulation of uterine growth was associated with increased 125I-EGF binding to uterine membranes (32) and increased levels of an EGF precursor protein in uterine tissue (33). This up-regulation of the EGF receptor was specific for estrogen treatment and was associated with a 3–4-fold increase in EGF receptor mRNA steady state levels (34). This observation raises the possibility that estrogen-induced uterine growth may be mediated, at least in part, through an autocrine pathway involving the EGF receptor. Murphy et al. (35, 36) have shown that progestin treatment of T-47D human breast cancer cells results in an increase in EGF receptor levels along with an increase in EGF receptor mRNA steady state levels. Thus steroid hormones, including estradiol, can mediate the heterologous up-regulation of EGF receptor and/or EGF-related factor production along with increased growth.

In liver, Earp and O'Keefe (37) have shown that a decrease in EGF receptor number occurs during regeneration. However, this decrease in EGF receptors is associated with an increased responsiveness of hepatocytes isolated from regenerating liver to EGF-induced DNA synthesis (38) indicating a lack of correlation of receptor number with EGF responsiveness. Along similar lines, Cruise et al. (39) have shown a strong synergistic interaction between norepinephrine and EGF resulting in enhanced DNA synthesis in cultured rat hepatocytes. This interaction was observed when norepinephrine and EGF were added together and, perhaps more significantly, when the hepatocytes were pretreated with norepinephrine for 24 h (40). Furthermore, the potentiation of EGF-induced DNA synthesis was associated with a decrease in 125I-EGF binding at 4°C which is
consistent with a decrease in cell surface EGF receptors (41). This is another example of the paradoxical findings that some agents which seem to down-regulate the EGF receptor, at the same time increase the growth responsiveness of cells to EGF. On the other hand, Lin et al. (42) reported that \( \mu \)M concentrations of hydrocortisone and \( \nu \)M concentrations of dexamethasone caused 70 to 100\% increases in EGF receptor levels in primary cultures of rat hepatocytes. These effects were detectable at 4 h and were inhibited by insulin. However, no data were presented on the glucocorticoid effects on induction of DNA synthesis by EGF.

Several recent studies have examined the effects of tumor promoters on liver EGF receptors. Hwang et al. (43) reported that feeding rats diet containing 0.05\% PB for 1 month caused decreased \( ^{125} \)I-EGF and \( ^{125} \)I-insulin binding to rat liver Golgi fractions which persisted through 5 months. No changes in affinities were detected and changes in receptor autophosphorylation were parallel to changes in ligand binding. Gupta et al. (44) reported decreased \( ^{125} \)I-EGF binding to hepatocytes isolated from rats fed 0.05\% PB, a CD diet or a PB + CD diet for 10 and 28 days. The decrease in receptor number was greater and occurred sooner in rats fed PB + CD. The dissociation constant of the receptor was decreased in all groups. Decreased EGF receptor number, but not \( K_d \), was also detected beginning after 10 days of feeding rats diet containing carcinogenic peroxisome proliferators (45). Finally, Eckl et al. (46) have shown that chronic treatment of hepatocytes with PB caused a decrease in hepatocyte EGF receptors and altered the proliferative response of hepatocytes isolated from PB-treated rats to media calcium concentrations.

Taken together, these results demonstrate that chronic in vivo treatment with liver tumor promoters causes down-regulation of insulin and EGF receptor numbers. In some cases this was accompanied by an increase in receptor \( K_d \). Decreased receptor numbers have also been found in liver nodules and carcinomas appearing during carcinogenesis mediated by the Solt-Farber protocol (47). Similar changes in EGF receptors have been associated with increased responsiveness to EGF, they may reflect a state of chronic growth stimulation mediated through the EGF receptor by EGF itself or a growth factor in the EGF/TGF\( \alpha \) family.

Treatment of cultured hepatocytes with tumor promoters has also been observed to affect the EGF receptor. Sawada et al. (17) examined the effects of TPA and PB on DNA synthesis and EGF responsiveness in cultured rat hepatocytes. They found that both agents caused small increases in DNA synthesis. However, pretreatment with TPA (50 ng/ml) or PB (1 mM) enhanced, by 60–80\%, the subsequent DNA synthetic response of hepatocytes exposed to EGF from 12 to 24 h. Pretreatment with these promoters enhanced DNA synthesis at all concentrations of EGF examined, but did not change the shape of the EGF dose-response curve. These results are very similar to what we observed in the present study with EE. Sawada et al. (17) also showed that TPA caused a decrease (approximately 44\%) in EGF binding within 2 h whereas PB had no initial effects. Subsequently, EGF binding increased to within 90\% of control and to 120\% of control values by 12 h after addition of TPA or PB, respectively, the time when EGF was added in experiments demonstrating potentiation of DNA synthesis. We observed similar results in studies with both TPA and PB on EGF-induced DNA synthesis and on EGF receptor levels (data not shown). These results are somewhat different from what is observed with EE. While EE pretreatment caused a potentiation of the DNA synthetic response to EGF, this potentiation was associated with a twofold increase in EGF receptor levels along with a small increase in receptor ligand affinity (decreased \( K_d \)).

In time course studies we saw no evidence of receptor down-regulation at early times after EE addition (data not shown). Thus, we agree with the conclusions of Sawada et al. (17) that EGF binding does not appear to be causally associated with the potentiation of EGF-induced DNA synthesis.

In conclusion, the results of our study, parts of which have been presented previously (48, 49), demonstrate that the liver tumor promoter EE has direct growth-related effects on rat hepatocytes. While treatment with EE alone caused only a relatively small stimulation of DNA synthesis, EE dramatically potentiated the DNA synthetic response to EGF and caused an increase in cell surface EGF receptor numbers. In other studies we have found increased levels of a protein factor(s) stimulatory for hepatocyte DNA synthesis in serum and plasma of female rats treated with EE (5). Together, these observations suggest the hypothesis that liver growth in response to EE treatment occurs through both direct and indirect effects on hepatocytes.

We are in the process of determining whether these two pathways intersect. Furthermore, we are engaged in studies to determine the mechanism by which EE enhances hepatocyte EGF receptor levels. In addition, since the twofold increase in receptor levels does not coincide with the dramatic potentiation of responsiveness to EGF, we are studying the effects of EE pretreatment on hepatocyte EGF receptor activity.

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