Effect of Tumor Promoting Contraceptive Steroids on Growth and Drug Metabolizing Enzymes in Rat Liver

Heike Ochs, Bernd Düsterberg, Peter Günzel, and Rolf Schulte-Hermann
Institut für Toxikologie und Pharmakologie der Universität Marburg/Lahn [H. O., R. S.-H.], and Research Laboratories, Schering AG, Berlin-Bergkamen, W. Berlin [B. D., P. G.], Federal Republic of Germany

ABSTRACT

Liver tumor formation in rats treated with oral contraceptive steroids for long periods has been associated with the tumor promoting potential of these agents. As other known liver tumor promoters, e.g., phenobarbital and hexachlorocyclohexane, induce liver growth and hepatic monooxygenases, we investigated whether or not estrogens have similar effects. Female rats were treated with a wide range of doses of ethinylestradiol, including human contraceptive doses, which are approximately 1 μg/kg. The physiological estrogen estradiol was studied for comparison. Also included were norethynodrel and norethisterone, which are approximately 1 μg/kg. These doses are below those used in previous tumor promotion studies in rats.

Using 5 different substrates to check monooxygenase activities of isolated liver microsomes, no induction or only very weak induction by estrogens was found.

These studies suggest that induction of liver growth may be a property relevant for the tumor promoting activity of estrogens; in contrast, induction of hepatic monooxygenases does not appear to be necessary for liver tumor promotion in the rat.

INTRODUCTION

Liver tumors may appear, albeit rarely, in women using oral contraceptive steroids (3, 4). In animal experiments with rats or mice, long-term treatment with relatively high doses of estrogenic or gestagenic steroids and their combinations resulted in the formation of liver tumors which were histologically classified as neoplastic nodules and hepatocellular carcinoma (see reviews in Refs. 5 and 6). The mechanisms underlying these carcinogenic effects are not clear.

Estradiol as well as various synthetic steroid estrogens have been classified as (complete) carcinogens by the International Agency for Research on Cancer on the basis of long-term animal studies (5). However, assays for mutagenicity, DNA damage, and DNA binding, which can indicate carcinogenic (initiating) activity or potential do not support this conclusion because they were negative with various estrogenic steroids (7–10). Furthermore, estrogens (i.e., estradiol and ethinylestradiol) failed to induce the appearance of putative preneoplastic lesions in rat liver (9, 11) in tests for tumor initiating activity.

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1 Some of the present results were published in preliminary form (1, 2).
2 Present address: Institut für Tumorbiologie-Krebsforschung der Universität Wien, Borschkegasse 8a, 1090 Wien, Austria. To whom requests for reprints should be addressed.

Received 6/17/85; revised 10/11/85; accepted 10/15/85.

The abbreviations used are: PB, phenobarbital; BPA, benzphetamine; CPA, cyproterone acetate; EE2, ethinylestradiol; EM, ethylmorphine; HCH, hexachlorocyclohexane; LI, labeling index; NET, norethisterone-norethindrone; NET-ac, norethisterone acetate; NET-en, norethisterone enanthate; PNA, p-nitroanisole; BSA, bovine serum albumin.

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Fig. 1. Effects of ethinylestradiol during a 7-day treatment period. Rats were treated with 0.5 mg EE2/kg body weight once daily (arrows). , EE2-treated animals; *, ad libitum fed controls; O, pair-fed controls. *P < 0.05; **P < 0.01; ***P < 0.001. In Section n, the number of hepatocytes and sinus wall cells per microscopic field was determined in histological sections. Columns indicate the quotient:

No. of sinus wall cells
No. of hepatocytes

as those applied in previous tumor promotion studies. Two human progestins that act preferentially as estrogens in rats, i.e., norethynodrel and NET (35), were also investigated.

MATERIALS AND METHODS

Animals and Treatment

Female Wistar rats (SPF) were obtained from Zentralinstitut für Versuchstierzucht, Hannover, Germany. They were 7-9 weeks old and weighed 140-180 g at the start of treatments. Animals were housed 5 per cage in a climatized room under controlled light-dark rhythm (lights on from 9:00-21:00, off from 21:00-9:00). In the experiment shown in Fig. 1 this rhythm was reversed (lights from 21:00 to 9:00) during 3 weeks before treatment for technical reasons. When EE2 and estradiol were administered orally via the diet (see below) rats were adapted during 3 pretreatment weeks to continuous lighting. This was done to eliminate the large fluctuations in food (and steroid) uptake associated with the circadian rhythm. Continuous lighting does not significantly affect the parameters studied. A pelleted rat chow (Altromin 1324, Altroge, Lage, Germany) was provided ad libitum unless stated otherwise. Water was always available.

All steroids investigated were from Schering AG. The identity and purity of the agents had been confirmed analytically. Purity was as follows: EE2, 98.9%; estradiol, 99.7%; norethynodrel, 98.9%; NET, 99.1%; NET-ac, 98.7%; and NET-en, 100%. In all experiments 5 rats were used for each control or treatment group. For s.c. treatments agents were dissolved in castor oil:benzyl-benzoate, 3:2. Concentrations were adjusted so that maximally 2 ml vehicle/kg were injected. Control animals received pure vehicle or remained untreated; no significant effect of the vehicle on the parameters studied was found. For oral treatments steroids were premixed with lactose and then admixed to a powdered

diet (Altromin 1321). Food consumption was measured daily, and the
steroid dose applied was calculated. Since the estrogens reduced food
consumption additional control rats were pair-fed. These animals re-
ceived their daily ration once daily at the beginning of the light phase.
In the experiment shown in Fig. 1, [methyl-3H]thymidine (0.2 mCi/kg)
(6.5 Ci/mmol; NEN, Frankfurt, Germany) was injected into a tail vein 1 h
before sacrifice. All animals were killed by decapitation at the onset of
the light phase. Blood was collected for determination of serum hormone
levels. The liver was quickly excised, blotted, and weighed. Microsomes
were prepared from fresh liver; specimens for other biochemical assays
were stored at -15°C. Liver samples for histological investigations were
fixed in formalin (10%). Perirenal adipose tissue from both sites was
excised and weighed as quickly as possible.

Biochemical Assays of Liver Composition

DNA, RNA, and Protein. Liver specimens were homogenized in 10
volumes of cold 2% perchloric acid with 0.1 mM EDTA. After centrifugation
the sediment was washed twice in cold 2% perchloric acid. DNA and
RNA were extracted into 5% perchloric acid at 80°C for 15 min. DNA
was then determined according to the method of Burton (38), and RNA
was determined according to the method of Fleck and Munro (37). Where
required the 3H-content of the extracts was assayed in a liquid scintillation
counter and calculated as dpm/μg DNA. Protein was measured as
described by Lowry et al. (38).

Glycogen. Liver specimens were extracted with water at 100°C. After
centrifugation free glucose was determined in the supernatant by means of
hexokinase (Boehringer test 124338). Glycogen present in the super-
natant was hydrolyzed with amyloglucosidase, and glucose was assayed
as above.

Microsomal Enzymes

Liver specimens from each treatment group were pooled, homo-
genized in 5 volumes of 0.25 M sucrose, and buffered to pH 7.4 with
Tris. The microsomal fraction was obtained by differential centrifugation
at 10,000 and 100,000 × g, and it was stored prior to use at -15°C. This
does not affect enzyme activities as shown in separate experiments.
Incubation mixtures contained, in a final volume of 0.5 ml: NADP (5 ×
10⁻⁴ M); isocitrate (5 × 10⁻⁵ M); isocitrate dehydrogenase (20 μU); MgCl₂
(3 × 10⁻³ M); and one of the following substrates: aminopyrine (10⁻³ M);
EM (5 × 10⁻⁴ M); BPA (1 × 10⁻⁵ M); aniline-HCl (2 × 10⁻⁵ M); and PNA
(0.5 × 10⁻⁵ M). All substances were dissolved in phosphate buffer (pH
7.4, 0.067 M). The samples were incubated for 20 min in a shaking water
bath at 37°C. Enzyme reaction was stopped by transfer to an ice bath
and addition of trichloroacetic acid (1.8 M, 250 μl). Formaldehyde for
fixation was added (1 M, 250 μl). The mixture was centrifuged (5000 × g
for 15 min) and the sediment was washed twice in cold 2% perchloric acid.
DNA, RNA, and Protein. Liver specimens were embedded in paraffin. Sections 5 μm thick were
cut and stained with hematoxylin-eosin, and autoradiography was performed
with Kodak Foto-emulsion NTB 3. The percentage of labeled
cells (LI) was determined for hepatocytes (LI-hepatocytes) and for sinus
wall cells (LI-sinus wall cells); the percentage of hepatocytes in mitosis
was also determined. At least 2000 hepatocytes were counted in each

Hormone Levels in Serum and Liver

Ethinylestradiol

Effect on Liver Growth: Time Course. Female rats received
0.5 mg EE₂/kg body weight daily s.c. for up to 7 days. Results
are shown in Fig. 1.

Body weights remained constant while ad libitum fed controls
increased their weight by 10% during the observation period
(Fig. 1a). Food intake decreased (Fig. 1c). This effect was more
pronounced in the first days of treatment. As an average, food
consumption of EE₂ treated rats was 73% of controls. The mass
of perirenal adipose tissue decreased (Fig. 1c).

Liver weight had increased by 21% after treatment for 7 days;
apparently the maximal increase was not yet reached at this
time point (Fig. 1d). DNA and RNA contents of the liver were
enhanced by 32 and 33%, respectively. Liver protein increased
by 14%. Glycogen content of the liver decreased in the first days
but had normalized at the end of the treatment period (Fig. 1, e–
h).

DNA synthesis was measured biochemically and autoradi-
ographically by means of [3H]thymidine. With both methods an
increase was found (Fig. 1, i and k). The greatest effect was
seen on day 1 (increase of 7- or 20-fold, respectively). The
percentage of hepatocytes in mitosis was enhanced by a similar
factor (Fig. 1f). The maximal effect was seen on days 1–3. Sinus
wall cells also showed an enhanced activity of DNA synthesis.
The slope of the increase was somewhat less pronounced than
with parenchymal cells (Fig. 1m). All parameters were still some-
what increased over control levels on day 7, indicating that the
growth process of the liver was not fully completed at this stage.
The sinus wall cell:parenchymal cell ratio did not change (Fig. 1n).

We conclude from these results that EE₂ induces liver growth
mainly by hyperplasia of hepatocytes. Sinus wall cells appeared

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to participate proportionally with parenchymal cells in the growth process. In an attempt to assess the possible contribution of the reduced food intake to the changes in EE2 treated rats we introduced pair fed animals as a further control. The effect of pair feeding on body weight was more pronounced than that of EE2 treatment (Fig. 1a). Perirenal adipose tissue decreased to the same extent as in EE2 treated animals (Fig. 1c). Weight as well as RNA and protein contents of the liver decreased in pair fed animals, while the DNA content remained unchanged (Fig. 1, d-g). DNA synthesis decreased below the level of ad libitum fed controls (Fig. 1, i and k). In contrast to EE2 treated animals the pair fed controls showed a complete depletion of glycogen in the liver throughout the treatment period (Fig. 1h). To some extent these results were probably due to the feeding schedule used. We noted that unlike EE2 treated rats the pair fed controls ate their daily ration within a few hours and were in a fasting state at the time of sacrifice. Nevertheless it seems obvious that the effects of EE2 treatment on the liver cannot result from reduced food consumption. Although both pair-fed and ad libitum fed controls were used in most of our experiments we consider the latter more reliable for interpretation of the results obtained, and we report only those in the following.

Concentration of EE2 in Liver and Serum. We studied EE2 levels following the seventh s.c. injection of the hormone. This schedule was used to check whether the hormone levels obtained by s.c. application are fairly constant throughout a 24-h period of the late phase of the experiment. As shown in Fig. 2, EE2 levels were 4-5 times higher in liver than in serum. There was a moderate increase within the first 4 h after treatment, followed by a plateau. Levels on day 7 were higher than on day 6, suggesting that a steady state of organ concentrations was not yet reached. This is consistent with the observation that liver growth did not seem to be at its maximum on day 7 of EE2 treatment (see Fig. 1).

Subcutaneous versus Oral Application. Shown in Fig. 3 is a comparison of the effects obtained after oral or s.c. application of EE2 for 7 days. At the higher doses tested EE2 when administered orally increased liver DNA, but it was somewhat less effective than after s.c. application, producing increments of 7 and 23% compared to 24 and 30% after s.c. treatment with identical doses. As after s.c. injection of EE2 (Figs. 1 and 3b) in animals fed EE2, liver weight increased relatively less than liver DNA (compared to the ad libitum fed controls). In fact, the liver weight increase was not significant in the present experiment (Fig. 3a). Thus induction of liver growth by oral EE2 would not have been detected without DNA and protein determination.

Dose Response Relation. Also shown in Fig. 3 is the result of experiments in which different doses of EE2 were applied for 7 days. Hepatic effects studied appeared to increase linearly with the logarithm of dose. As after s.c. injection of EE2 (Figs. 1 and 3b) in animals fed EE2, liver weight increased relatively less than liver DNA (compared to the ad libitum fed controls). In fact, the liver weight increase was not significant in the present experiment (Fig. 3a). Thus induction of liver growth by oral EE2 would not have been detected without DNA and protein determination.
for induction of liver growth via the s.c. route; 5-10 μg EE2/kg were obtained with the oral route.

**Microsomal Monooxygenases.** The activity of hepatic drug metabolizing enzymes was tested *in vitro* with 5 different substrates using isolated microsomes. It was found that EE2 treatment did not affect the turnover of the substrates to a great extent (Table 1). The demethylation of EM and BPA was somewhat enhanced in a dose-dependent manner, but it should be noted that these stimulatory effects are much smaller than the several-fold increases produced by steroids carrying a C20-C21 side chain (54, 55). Furthermore, the metabolism of aniline and PNA even tended to be depressed after s.c. treatment (Table 1). (Since the microsomes were obtained from pooled livers, SD and P-values could not be calculated. However, we have performed 5 independent experiments with s.c. EE2 and found the results shown in Table 1 to be well reproducible.)

### Table 2

| Animals were kept under continuous lighting; they had free access to estradiol-containing food until 2 h before decapitation. |
|---|---|---|---|---|
| Dose (mg/kg/day) | Controls fed | Controls pair-fed | Serum (ng/ml) | Liver (ng/g) |
|controls fed | ad libitum | 0.08 ± 0.02 | 0.6 | 0.040 ± 0.002 | 0.05 ± 0.01 | 0.6 |
| 0.05 ± 0.002 | 0.05 ± 0.01 | 0.6 |
| 0.36 ± 0.02 | 0.08 ± 0.02 | 0.6 |
| 3.15 ± 0.37 | 0.21 ± 0.08 | 8.4 ± 4.1 |

*Mean ± SD.

**Norethynodrel.** Application of norethynodrel decreased body growth and food intake, which is consistent with its estrogenic action in the rat (35). S.c. injection increased liver weight as well as DNA and protein content in the liver and, as after EE2, the percentage of increment of DNA was more pronounced than that of liver mass. Oral administration did not appear to be effective at the doses used (Fig. 5).

**Norethisterone and its Acetate and Enanthate Esters.** Fig. 6 shows the effect of NET and its fatty acid esters NET-ac and NET-en. All three compounds decreased body growth and food intake (data not shown) and increased liver weight and DNA content. These effects appeared to depend on the dose with NET and NET-ac. The small efficacy and lack of dose dependence seen with NET-en may be due to the slow release of this agent from its s.c. depot. Thus, the half-life of NET in blood serum is 13 days after s.c. application of NET-en (35), but it is 14 h after a s.c. dose of NET (44).

**Microsomal Monooxygenases.** Treatment with estradiol,
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NET, or NET esters did not cause distinct rises in the turnover of the substrates used. In contrast, aniline and PNA metabolism were slightly depressed (Table 3).

**DISCUSSION**

Our results show that the estrogenic steroids tested are able to induce liver growth in female rats. This was demonstrated by increases of relative liver weight and of DNA, RNA, and protein contents of the liver. As shown with EE2, the increase of DNA is due to enhanced parenchymal DNA and cell replication. These results confirm and extend a few previous studies. Schwarzlose and Heim (45) reported induction of liver growth by estradiol and female mice. After completion of our study a recent report by Fisher et al. (46) confirmed this finding for rats. Sweeney and Cole (47) and MacKinnon et al. (48) noted liver enlargement in rats after EE2, and Mitznegg et al. (49) described liver growth in rats after treatment with a combination of EE2 and norgestrel.

Unlike virtually all other chemical stimuli of liver growth studied...
so far (32, 33, 50) the estrogenic steroids tended to increase liver DNA relatively more than liver mass or protein. As shown histologically with EE2 this excessive DNA increase did not appear to result from over-proportional multiplication of (small) non-parenchymal cells, as suggested by others (51). The unusually small increment of liver mass (compared to DNA) could explain why up to now only a few authors took notice of the induction of liver growth by estrogenic steroids. A further explanation may be that the absolute liver weight increases less than relative weight because body growth of estrogen treated rats is delayed (present study; 52).

Whereas previous studies (45-49) on estrogen-induced liver growth used relatively high doses, the present work shows that the extrapolated threshold dose of estradiol or EE2 is as low as 1 µg/kg; a dose of 2 mg/kg EE2 leads to an increase of liver DNA by 60%. Therefore, EE2 is one of the most potent inducers of liver growth found so far. These observations lend support to previous hypotheses (45, 46) suggesting that endogenous estrogens may be involved in the regulation of liver size in normal and, particularly, in pregnant rats. Furthermore, Lombard et al. (53) suggested that endogenous estrogens accelerate hepatic DNA synthesis in response to partial hepatectomy.

Unlike many other hepatomitogens with steroid or non-steroid structure (32, 33, 54), the 4 different rat estrogens studied here caused no increase or only weak increases of hepatic monooxygenases. Of the enzyme activities tested EM demethylation showed the most pronounced and reproducible increase of 40-80%. This is a small effect compared to 4-8-fold increases seen after doses of pregnenolone-16α-carbonitrile, CPA, or PB which are approximately equipotent in the induction of liver growth (50, 55). Likewise, other authors found no increase of EM or BPA demethylation after estrogen treatment of female rats (56) or even decreases of EM demethylase and cytochrome P₄₅₀ in male rats (48, 57). Furthermore, P₄₅₀ dependent enzymes were found to be depressed in male rats treated with EE2 (47). These studies do not rule out the possibility that estrogens might induce a peculiar monooxygenase with little affinity to any of the various substrates used in the present and previous studies. Nevertheless, it seems safe to conclude that estrogens differ from other hepatomitogens and tumor promoters (e.g., PB, HCH, and CPA) in that they induce liver growth without an appreciable increase in the monooxygenase activities tested here.

In the adult organism liver growth is usually associated with enhanced functional performance (32, 33), or it is of regenerative nature. Since the low doses of estrogen studied are not known to cause hepatocyte injury and death which could give rise to regenerative liver growth, one might expect some functional increases. In fact a few hepatic functions are known to be increased by estrogens, e.g., synthesis and secretion of renin substrate (58), clotting factors (59), and globulins binding T₄ or cortisol (60, 61) and binding and internalization of low-density lipoprotein (62); in avian liver, estrogens trigger an enhanced synthesis of vitellogenin (63). To understand the relationship of these estrogen effects to each other and to liver growth may be important for further studies on the mechanism of tumor promotion (overexpression hypothesis; see below).

Liver tumor promotion by estrogens was reported by Taper (14) and Yager et al. (17) with different initiating carcinogens and different estrogen, i.e., long-acting estradiol-esters, EE₂, and mestranol. The promoting activity of EE₂ and mestranol was confirmed by using growth of putative preneoplastic (γ-glutamyltranspeptidase-positive) foci and neoplastic nodules as the endpoint (15-17, 19). The synthetic estrogen diethylstilbestrol was also found to promote liver tumorigenesis (15). On the other hand Mishkin et al. (64) reported inhibitory effects of estradiol on the extent of γ-glutamyltranspeptidase-positive areas in the liver and on formation of malignant tumors. Their schedule of initiation, however, differed from that in other studies in that acetylaminofluorene was administered in 4 cycles up to the stage of appearance of acetylaminofluorene independent nodules before estrogen treatment was started. Possibly, therefore, the promoting effect of estrogens is restricted to early stages of neoplastic development in the liver, which would be consistent with promotion by PB and HCH.

A major aim of the present study was to check tumor promoting estrogens for early effects on the liver that might be relevant for tumor promotion. It seems clear that pronounced induction of certain hepatic monooxygenases as observed after PB, HCH, CPA, or methylcholanthrene is not a prerequisite for tumor promotion by estrogens. However, estrogens share the ability to induce liver growth with virtually all other liver tumor promoters that lack distinct cytotoxic effects in this organ. Furthermore, estrogen doses found to have tumor promoting activity (14-17, 19) were well above the “threshold dose” estimated in the present study for induction of liver growth. This may also hold true for mestranol (16, 17), which was not studied by us but which is known to be a “pro-drug” activated to EE2 in vivo by 3-O-demethylation (65). Therefore it seems likely that the estrogens used produced some hepatic hyperplasia in the above promotion studies. This lends support to our hypothesis that the ability to induce liver growth is one of the properties necessary for liver tumor promotion by non-cytotoxic drugs.

It must be emphasized here that tumor promotion appears to be a complex process not explicable simply by growth stimulation in the target organ. Rather, tumor promotion may result from activation of specific functional (and gene) programs, which are overexpressed in preneoplastic cells (31, 34). Different promoters may activate different programs that share, however, as a common component the potential to trigger liver growth.

This concept could provide a convenient pre-test for detection of chemicals with tumor promoting activity or potential. Induction of liver growth can be measured within a few days and even in vitro with isolated hepatocytes (66) and thus requires much less time and effort than studies utilizing growth of altered foci or of tumors as endpoints. Induction of liver growth could therefore be checked at an early stage of toxicological testing before more specific tests are initiated.

Finally, human contraceptive doses of EE₂ and mestranol are between 30 and 100 µg, i.e., similar to the “threshold dose” extrapolated in the present study for induction of liver growth by EE₂ in rats. This could support the hypothesis that the occurrence of adenomas in human liver under contraceptive steroid medication may result from a promoting effect on a rare preneoplastic cell. However, to avoid any premature extrapolation to humans from the present “threshold dose” in rats it has to be emphasized that profound differences exist between rats and humans with respect to elimination pathways and metabolic clearance of EE₂ (67). Moreover, in comparison to rats the sensitivity of human liver cells for EE₂ with regard to growth promotion is not yet known. To our knowledge the effects of...
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EE₂ on the liver described in the present study, specifically the induction of growth and DNA synthesis, have not yet been described for human livers, even after long-term usage of higher dose levels of EE₂.

**REFERENCES**


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