Quantitative Structure-Activity Studies on Effects of Sixteen Different Steroids on Growth and Monooxygenases of Rat Liver

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ABSTRACT

Sixteen steroids with different endocrine activities were administered to female rats for 6 or 7 days, in a broad range of doses. Liver growth was recorded by measuring weight and DNA contents and monooxygenase activity by assaying the turnover of five different substrates. According to their effects on these parameters steroids were assigned into one of the following three groups: (a) Estrogens estradiol and ethinylestradiol, as well as the progestins norethynodrel and norethisterone (norethindrone) which have estrogenic activity in rats. These agents induced pronounced liver growth and excessive DNA increase which was not associated with major monooxygenase induction. (b) A different type of response consisted of liver growth and DNA increase associated with a pronounced induction of monooxygenases(s) in a characteristic pattern. This response was elicited by pregnenolone-16 α-carbonitril, by progestins progesterone, cyproterone acetate, and medroxyprogestosterone (but not gestodene and levonorgestrel), by the antimineralocorticoid spironolactone and by the glucocorticoids cortisol and dexamethasone. Apparently, this response pattern was not related to any specific endocrine action but to certain structural features, in particular to the presence of a saturated, at least two-membered alkyl substituent at C17 of the steroid ring system. (c) No or small effects were observed after gestodene, levonorgestrel and the androgens testosterone and methyltestosterone.

Dose response studies revealed that estrogens estradiol and EE2 induced hepatic effects more potently by four orders of magnitude than progestins. The response patterns observed may be relevant to the tumor-promoting activity of some of the steroids tested.

INTRODUCTION

Long-term use of contraceptive steroids in rare cases may result in tumor appearance in human liver. Such tumors usually seem to be benign and in some instances regressed after withdrawal of steroids (1–3). No evidence of genotoxic or tumor-initiating activity of the steroids has been detected (3–7). It has therefore been assumed that tumor appearance in human liver under contraceptive medication is due to a promoting effect on the growth of accidental preneoplastic lesions.

Experiments on rodent animals have clearly supported the possibility of a tumor-promoting action of certain contraceptive steroids. This was found with both estrogens (8–11) and progestins (12, 13). So far it is not known whether the promoting activity is due to specific endocrine or structural properties of the steroid molecules. However, in recent years evidence has accumulated suggesting that many liver tumor promoters share the ability to induce growth in this organ. This has been observed with progestins (14, 15) and estrogens (16, 17) and also with nonsteroid tumor promoters such as phenobarbital, hexachlorocyclohexane, dichlorodiphenyltrichloroethane, polychlorinated biphenyls carbazine, tetrachlorodibenzop-dioxin, etc. (18, 19).

Liver growth observed after application of these agents does not appear to occur as a response to tissue damage as such damage is usually absent. On the other hand, liver growth frequently is associated with functional increases such as of drug-metabolizing monooxygenases and is therefore considered an adaptive response.

The apparent association of liver tumor promotion with growth and monooxygenase induction in this organ made it worthwhile to investigate whether any relation may exist between these hepatic actions and structural or endocrine properties of the steroids. Therefore we have studied the effects of 16 different steroids with diverse chemical structures including estrogens, gestagens, antiandrogens, glucocorticoids, and an antimineralocorticoid (Fig. 1). Where feasible several doses of the steroids were tested for quantitative comparisons of their efficacy and for estimation of NOEL. Some results obtained with single steroids have already been published (14–16, 20, 21).

In this paper we present a compilation of the observations made with all of the 16 agents. The results suggest that according to their hepatic effects the steroids can be grouped into three different classes which may be distinguished by endocrine and structural properties.

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 treatment. Animals were housed five per cage in a climatized room under controlled light-dark rhythm (lights on from 0900–2100, off from 2100–0900). In a few experiments these lighting conditions were somewhat modified as follows: continuous lighting, Figs. 3 (E2) and 5 (progesterone); inverted light-dark rhythm, Fig. 2 (EE2, CPA) and Figs. 3 and 5 (PCN). Various control experiments have shown that these modifications have no significant effects on the results obtained. A powdered or pelleted standard rat chow (Altromin 1321; Altroge, Lage, Germany) was provided ad libitum. Tap water was always available.

All steroids investigated were obtained from Schering AG (Berlin, Germany). The identity and purity of the agents had been analytically confirmed; purity was greater than 98% in all cases. At least five rats were used for each control or treatment group. For s.c. treatment agents were dissolved in castor oil/benzylbenzoate (3:2). Concentrations were adjusted to inject maximally 2 ml vehicle/kg. CPA was dissolved in corn oil (0.4%) to administer 10 ml/kg, PCN was suspended in water; both agents were administered orally by gavage. Control animals received pure vehicle or remained untreated, no significant effect of the vehicle on the parameters studied was found (22). Other steroids to be given orally were premixed with lactose and then admixed to powdered diet. Food consumption was measured daily, and the steroid dose applied was calculated. Animals were treated for 7 days at the onset of the light phase, killing by decapitation was at the same time on Day 8. 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 (4%).

3 The abbreviations used are: NOEL, no observed effect level; AP, aminopyrine, AN, aniline; BPA, benzphetamine; CPA, cyproterone acetate; E2, estradiol EE2, ethinylestradiol; EM, ethylmorphine; LI, labeling index; PNA, p-nitroanisole; PCN, pregnenolone-16α-carbonitril.
Biochemical Assays of Liver Composition. Measurements for DNA, RNA, and protein are as follows. Liver specimens were homogenized in 10 vol cold 2% perchloric acid with 0.1 M 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 Burton (23), and RNA according to Fleck and Munro (24). Where required the \(^3\)H content of the extracts was assayed in a liquid scintillation counter, and calculated as dpm/\(\mu\)g DNA. Protein was measured as described by Lowry (25).

Microsomal Enzymes. Liver specimens from each treatment group were pooled and homogenized in 5 vol sucrose (0.25 M), buffered to pH 7.4 with tris. The microsomal fraction was obtained by differential centrifugation at 10,000 and 100,000 \(\times\) g, and was stored prior to use at \(-15^\circ\)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 \(\times\) 10\(^{-4}\) M), isocitrate (5 \(\times\) 10\(^{-3}\) M), isocitrate dehydrogenase (20 mU), MgCl\(_2\) (3 \(\times\) 10\(^{-3}\) M), and one of the following substrates: AP (10\(^{-2}\) M), EM (5 \(\times\) 10\(^{-3}\) M), BPA (10\(^{-3}\) M), AN (2 \(\times\) 10\(^{-3}\) M), pNA (0.5 \(\times\) 10\(^{-3}\)). All substances were dissolved in phosphate buffer (pH 7.4, M/15). The samples were incubated for 20 min in a shaking water bath at 37°C. Enzyme reaction was stopped by transfer into an ice bath and addition of trichloroacetic acid (1.8 M, 25 \(\mu\)l). Formaldehyde formation from AP, EM, and BPA was measured according to Nash (26), pNA O-demethylation was determined by measuring \(p\)-aminophenol formation (27) and AN metabolism by measuring \(p\)-aminophenol formation (28). All assays were conducted in triplicate.

Histological Procedures. Liver specimens were embedded in paraffin. Sections, 5-\(\mu\)m thick were cut, stained with hematoxylin-eosin, and autoradiography was performed with Kodak foto emulsion NTB3. 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 liver.

Statistics. Means obtained from five rats and standard deviations are given. The significance of differences was checked by Student's \(t\) test. Some of the results are expressed in percentage of controls from the respective experiments for the purpose of clarity. Statistical significance of steroid-induced changes was always checked against the controls from the respective experiments using the absolute data. A correction for multiple application of the test method to the same data (e.g., Bonferoni correction) was not performed.

RESULTS

Liver Growth. Of the series of steroids tested the estrogen EE\(_2\) and the progestin CPA were found to be the most potent inducers of liver growth. Effects of these two agents were
structure-activity studies on hepatic effects of steroids

... therefore investigated in greater detail and are displayed for comparison in Fig. 2. 0.5 mg/kg EE2 and 40 mg/kg CPA were administered s.c. and orally, respectively. As shown EE2 increased liver size by 21% and liver RNA and DNA by 33%; CPA enhanced these three parameters by approximately 40% (Fig. 2). Both agents stimulated hepatic DNA synthesis as shown by biochemical and autoradiographic determination of [3H]thymidine uptake into DNA and nuclei, respectively (Fig. 2). The pronounced increases of hepatocyte LI and mitotic activity (Fig. 2) indicate that parenchymal hyperplasia is an important factor in the overall liver enlargement. In addition sinus wall cells showed an enhanced LI and therefore seemed to participate in the growth process (4, 6). It should also be noted that both agents increased DNA synthesis only transiently; apparently, at the end of the treatment period of 7 and 6 days, respectively, liver size and DNA approached a new steady state at an enhanced level. In summary, we conclude from these findings that both EE2 and CPA induce ordered growth in female rat liver.

In further studies we determined liver mass and DNA as parameters representative of liver growth and hyperplasia. The results of dose-response studies with the various steroids were expressed as percentage of controls to facilitate comparisons and are shown in Fig. 3. For the sake of clarity, steroids were grouped according to their hepatic effects into three different classes in Fig. 3 and following figures.

Those steroids which act predominantly estrogenic in rats (group 1: estradiol, EE2 norethynodrel, and norethisterone) proved to be potent inducers of liver growth. Liver weight as well as DNA content increased considerably by up to 50%; remarkably, the relative increase of DNA was always more pronounced than the increase of liver mass. The effects were clearly dose dependent. NOELs were estimated graphically by extrapolation of the growth-response curve to the abscissa and were found to be in the order of 0.001 mg/kg for E2 and EE2 and 0.5–7 mg/kg for norethisterone, respectively.

Treatment with progesterone, CPA, OH-CPA, and spironolactone (group 2) produced dose-dependent increases in relative liver weight as well as in DNA content. The same effects were seen with PCN (not shown, see Refs. 14 and 20). Percentage increases in DNA were smaller than or, at best, similar to percentage increases of liver weight. Extrapolated NOELs were 100 mg/kg (progesterone) and 5–10 mg/kg (CPA). NOELs of these two progestins were thus approximately four and five orders of magnitude higher than those of estradiol and EE2; on the other hand, dose-response kinetics appeared to be steeper with the progestins (Fig. 3).

Hydrocortisone and dexamethasone also enhanced liver weight and DNA when related to 100 g body weight suggesting that these steroids have some potential to induce liver growth. However, this observation should be accepted with care because both glucocorticoids led to a marked decrease in body weight of the animals (Fig. 4). In fact, absolute liver weights remained about constant, and DNA or protein per total liver actually decreased. Thus, there was probably no or little true liver growth during glucocorticoid treatment.

The steroids listed in group 3 (gestoden, levonorgestrel, testosterone, and methyltestosterone) exerted small or no significant effects on relative liver weight and DNA content (Fig. 3).

Liver microsomal monooxygenases. Microsomal enzyme activity was studied by measuring the turnover of five different substrates by isolated liver microsomes. Results obtained with treated animals were expressed in percentage of controls (columns in Fig. 5). Of the estrogens and progestins with estrogenic activity in rats as presented in group 1 estradiol and norethynodrel exerted hardly any influence on the turnover of the test substrates. EE2 and norethisterone had a small effect on hepatic monooxygenases. Of these, EM demethylation was increased most.

The steroids listed in group 2 caused a pronounced increase of microsomal enzyme activities. After treatment with PCN an 8-fold increase in EM-demethylation was seen. Demethylation of AP and BPA was enhanced by a smaller factor while AN and pNA turnover remained nearly unchanged. This pattern was seen after treatment with CPA and all other steroids listed in part 2 of Fig. 5, even though the extent of increases varied considerably from compound to compound.

In group 3, gestoden, levonorgestrel, and testosterone did not affect the monooxygenases tested or very weakly so, while methyltestosterone caused a moderate increase in N-demethylations.

In conclusion, these studies revealed two different patterns of monooxygenase alterations, one represented by the effects of EE2, the other by those of PCN/CPA. It is important to emphasize that these patterns proved to be highly reproducible.
in seven different experiments where CPA and EE2 were used as positive controls. It should also be noted that both patterns clearly differed from those induced by phenobarbital or 3-methylcholanthrene (Fig. 5).

When different doses of the steroids were used, again the monooxygenase induction patterns seen in Fig. 5 were obtained. For the sake of brevity we present in Fig. 6 only the results obtained with EM demethylation. They are expressed in percentage of controls. While there was no induction of EM demethylation after E2 treatment, EE2 produced a moderate,
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Fig. 5. Patterns of monoxygenase activities. Doses indicated were given once daily for 6 or 7 days. Columns indicate the turnover of test substrates by pooled microsomes in µg product/mg microsomal protein x 20 min expressed as a percentage of controls. Control values were: AP, 1.57 ± 0.45; EM, 0.76 ± 0.28; BPA, 1.19 ± 0.30; AH, 1.10 ± 0.27; pNA, 1.75 ± 0.53; Results with PCN, 3-MC, and PB are taken from Ref. 20.

Fig. 6. Ethylmorphin demethylation: Dose response relations with different steroids. Columns, turnover of EM by pooled microsomes from steroid treated animals in percentage of controls. Control values were 1.01 ± 0.20 µg H₂CO/mg microsomal protein/20 min.

Table 1 Summary of hepatic responses to the steroids tested

| Extents of increases over controls as obtained with the maximally effective doses are indicated in an approximate way by 0-+++.
<table>
<thead>
<tr>
<th>Liver growth</th>
<th>Enzyme pattern like</th>
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<td>Response type</td>
<td>organ enlargement</td>
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<td>I</td>
<td>Estradiol</td>
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<td>Norethynodrel</td>
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<td>Norethisterone</td>
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<td>Testosterone</td>
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<td>Methyltestosterone</td>
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DISCUSSION

We conclude from this study that the steroids investigated can induce at least two different types or patterns of response in the liver as summarized in Table 1: (a) Liver growth characterized by an excess of relative DNA increase over weight increase, associated with no or small monoxygenase increases of the "EE₂ pattern." (b) Liver growth with relatively smaller or no increases of DNA, associated with pronounced monoxygenase increases of the "PCN pattern"; dose-response kinetics increased more steeply than in group 1. We have assigned the steroids to one of these groups. Steroids with small or no effects on liver growth and monoxygenases that could not unequivocally be assigned to groups 1 or 2 were assigned into a third group (type III) (Table 1).

Can these response types be correlated to any known endocrine or structural properties of the steroids? Group 1 comprises those four steroids of the present study that in rats act predominantly and strongly estrogenic at the effective doses (29). Consequently, we hypothesize that the responses of group 1 may be due to estrogenic activity of the agents. This conclusion is supported by inhibitory effects of clomiphene, an antiestrogen, on estrogen-induced liver growth and DNA synthesis (17, 30).

In contrast, group 2 responses do not appear to be correlated with any particular endocrine effect: PCN has virtually no
endocrine effects, progesterone, medroxyprogesterone, CPA and hydroxy-CPA are progestins, spironolactone is an antiimineralcorticoid, and cortisol and dexamethasone are glucocorticoids. On the other hand, progestins levonorgestrel and gestoden cannot be assigned to group 2. It is, however, remarkable that the steroids of group 2 share structural properties which are not found in groups 1 and 3, i.e., a saturated alkyl substituent with at least two carbon atoms at position 17 (Fig. 1). Apparently, this side chain may be hydroxylated at C17 or not, may be substituted at C17 in α (spironolactone) or β (other steroids) configuration, and can be part of a lactone. However, unsaturation of the side chain as in C17-ethinyl derivatives (groups 1 and 3) seems to cause inactivity.

All three types of response of the steroids investigated clearly differ from those of phenobarbital or methylcholanthrene. This observation is consistent with the results of previous studies (20, 31, 32).

In most earlier investigations hepatic effects of single or only a few steroids were reported. The results generally agree with those of the present work, e.g., induction of liver growth by estrogens in the absence of pronounced stimulation of monooxygenase activities (3, 33, 34). Testosterone and methyltestosterone did not induce distinct liver enlargement (35) but, unlike our observations, methyltestosterone had a distinct inducing effect on EM demethylation (36, 37); the reason of this discrepancy is not known. Induction of liver growth and/or of monooxygenases by PCN, CPA, spironolactone, medroxyprogesterone, and other members of our group 2 was also noted before (20, 32, 38-40).

An extensive structure-activity study with respect to monooxygenase induction was reported by Heuman et al. (31). These authors used an immunological assay to show that hepatic concentrations of the PCN-inducible cytochrome P450 increased following treatment of rats with spironolactone, cortisol, and dexamethasone, but not after ES, mestranol, testosterone, and methyltestosterone. In vitro studies using isolated hepatocytes suggested that induction of P450-PCN is mediated by a receptor mechanism unrelated to the "classical" glucocorticoid receptor (41). While these results strongly support our conclusion as to existence of group 2 of steroid effects, Heuman et al. (31) found no increase of cytochrome P450-PCN in rat liver in vivo after progesterone. This apparent difference from our study is probably due to route of application and dosage of progesterone used by those authors (50 mg/kg i.p. once daily) (see 31). Since progesterone has a short biological half-life this regimen may not provide liver levels sufficiently high for a sufficient period of time to induce monooxygenase(s).

With respect to liver tumor promotion it is of interest to note that most promoting steroids recognized by now would be classified in group 1 or 2 of the present investigation, i.e., estradiol and estradiol esters (8), EE2 (9-11, 42), CPA, progesterone (12, 13), cortisol, and dexamethasone (42). Testosterone slightly enhanced the number (but not the size) of γ-GT positive foci in rat liver (42); a combination of testosterone esters did no significant effect on liver tumor development in intact rats but was promoting in gonadectomized rats (43). Thus testosterone may have a weak tumor-promoting effect in rat liver, and this would be paralleled with the present observations where the compound although assigned to group 3 did produce some liver growth. In summary these observations seem to support our hypothesis that the ability to induce liver growth is an important property of hepatic tumor promoters, although a strict quantitative correlation between stimulation of liver growth and tumor promotion does not appear to exist (44, 45).

REFERENCES


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