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REGULATION OF CYTOCHROME P-450-DEPENDENT CATECHOL ESTROGEN FORMATION IN RAT LIVER MICROSOMES

EVIDENCE FOR INVOLVEMENT OF ESTROGEN RECEPTORS*

KATHLEEN T. SHIVERICK†‡ and MORRIS NOTELOVITZ§

†Departments of Pharmacology and Therapeutics and §Obstetrics and Gynecology, and The Center for Climacteric Studies, University of Florida, Gainesville, FL 32610, U.S.A.

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Abstract—Experiments were conducted to evaluate whether estrogen 2-hydroxylase activity in liver microsomes, the main pathway for oxidative metabolism of estrogens in the rat, is regulated by administration of synthetic estrogens. Ovariectomized rats were treated with ethinylestradiol (EE), 100 μg s.c. for 3 days. Liver microsomes from EE-treated animals showed a 2-fold increase over control in estrogen 2-hydroxylase activity measured over a substrate concentration range of 0.5 to 50 μM. Double-reciprocal plots of enzyme activity as a function of substrate concentration were linear; apparent V_{max} values were 2-fold greater in microsomes from EE-treated animals while apparent K_m values for control and EE preparations were not different. Administration of the triphenylethylene antiestrogen tamoxifen (TAM), 100 μg s.c. for 3 days, did not affect microsomal catechol estrogen formation activity, and apparent K_m and V_{max} values were comparable with controls. When EE and TAM were co-administered, no increase in microsomal estrogen 2-hydroxylase was observed, and apparent K_m and V_{max} values were not different from either control of TAM-treated preparations. Thus, acute administration of EE was associated with a specific increase in the apparent V_{max} of estrogen 2-OHase activity, and this effect was not observed when TAM was co-administered with the estrogen.

The oxidative metabolism of both synthetic and naturally occurring estrogens occurs in hepatic microsomes by cytochrome P-450-dependent enzymes [1, 2]. A major metabolic pathway *in vivo* and *in vitro* has been shown to involve 2-hydroxylation of the aromatic A ring to form catechol estrogen products [1–3]. Catechol estrogen metabolites formed *in situ* in the liver can interfere with catecholamine [4] and drug metabolism [5], as well as undergo further metabolism to reactive products which covalently bind to cellular proteins [6]. The possible role of catechol estrogens in the side effects of synthetic estrogens used in contraceptive steroid preparations has led us to investigate the hormonal regulation of the estrogen 2-hydroxylase pathway in liver microsomes from female rats.

Conflicting evidence exists relative to the role which estrogens play in the regulation of hepatic steroid hydroxylase reactions. Administration of estrogens to male rats dramatically inhibits microsomal hydroxylation of several C-19 and C-21 steroid substrates [7]. The extensive work of Gustafsson and his co-workers [8] has indicated that pituitary factor, termed feminotropin, maintains the low level of steroid hydroxylase activity in female microsomes. An exception to this inhibitory pattern is the estrogen-dependent cytochrome P-450 system which hydroxylates steroid sulfates, an activity present in liver microsomes from female, but not male rats [9]. This laboratory has reported recently [10] that catechol estrogen formation in liver microsomes was increased following administration of the synthetic estrogen ethinylestradiol to female rats. These observations may be consistent with recent data which emphasize the catalytic heterogeneity and multiplicity of liver microsomal cytochrome P-450 [11]. Thus, estrogens could inhibit most pathways of steroid hydroxylation while selectively enhancing activity towards physiologically important substrates. In the present study, experiments were performed using the triphenylethylene anti-estrogen tamoxifen in an effort to elucidate the mechanism by which ethinylestradiol stimulates liver catechol estrogen formation.

MATERIALS AND METHODS

Chemicals. Ethinylestradiol was obtained from Steraloids (Wilton, NH). Tamoxifen was a gift from Dr. D. McCurdy, ICI Americas, Inc. (Wilmington, DL). [^{3}H]*S*-Adenosyl-l-methionine (68 Ci/mmole) and [6,7-^{3}H]ethinylestradiol (50 Ci/mmole) were purchased from the New England Nuclear Corp. (Boston, MA). The following were from the Sigma Chemical Co. (St. Louis, MO): NADPH, ascorbic acid, catechol-*O*-methyl transferase (COMT) and mushroom tyrosinase.

Animals. Adult, female Sprague–Dawley rats (250 g) were housed in wire cages with food and water *ad lib.* in a room with a 7:00 a.m.–7:00 p.m.

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‡ Address reprint requests to: Dr. Kathleen Shiverick, Department of Pharmacology, JHM Health Center Box J-267, University of Florida, Gainesville, FL 32610, U.S.A.

light cycle. Animals were ovariectomized 2 weeks prior to treatment. Control animals were administered propylene glycol vehicle (0.2 ml); EE-treated animals received ethinylestradiol 100 μg/day, s.c., in propylene glycol for 3 days; TAM-treated rats received tamoxifen (free base), 100 μg/day, s.c., in propylene glycol for 3 days; EE + TAM animals received both ethinylestradiol and tamoxifen for 3 days.

Enzyme assay. Animals were killed by decapitation. The liver was excised immediately, and a 20% homogenate was prepared in 50 mM sodium phosphate (pH 7.4) containing 1.15% KCl (w/v) with a Potter–Elvehjem homogenizer. The microsomal pellet was prepared from the 9,000 *g* supernatant fraction by centrifugation at 105,000 *g* for 60 min. Microsomes were resuspended in 0.1 M KPO_4, pH 7.4, so that 1 ml contained microsomes from 1 g of liver.

Catechol estrogen formation was measured by a modification of the radioenzymatic method of Paul *et al.* [12]. Ethinylestradiol was used as the substrate because it is metabolized exclusively to catechol products [2], a factor which was essential for kinetic studies. The incubation mixture contained 0.1 M potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 0.5 mM ascorbic acid, 0.5 mM NADPH, [^{3}H]*S*-adenosyl methionine (0.5 μCi/50 nmoles), and 2 units of COMT in a final volume of 0.10 ml. Ethinylestradiol concentration was varied between 0.5 and 100 μM, and substrate was always added in 0.002 ml ethanol. Product was extracted as described by Paul *et al.* [12]. All activities were corrected for values in the absence of NADPH. Product identification required synthesis of 2-hydroxy [6, 7-^{3}H]-ethinylestradiol standard by incubating [6,7-^{3}H]-ethinylestradiol with mushroom tyrosinase [13]. The 2-hydroxy [^{3}H]ethinylestradiol product was then methylated with COMT and *S*-adenosyl methionine using the incubation system described above. The radiolabeled metabolite of ethinylestradiol formed by liver microsomes was characterized by TLC and found to comigrate as a single radioactive peak with the methylated 2-hydroxy [^{3}H]ethinylestradiol standard in the following systems: (A) cyclohexane–chloroform–acetic acid (40:50:10, by vol.) on silica gel plates, and (B) methanol–water (80:20) on C_{18} μBondapack reverse phase plates (Whatman).

Protein concentrations were measured by the biuret method [14]. Cytochrome P-450 was determined by the dithionite reduced CO-difference spectrum according to Omura and Sato [15].

Statistical analysis. Statistical comparisons were made by Student's *t*-test. Straight line plots in double-reciprocal graphs were fitted by linear regression analysis [16]. Apparent K_m and V_{max} values were derived for each microsomal preparation, with the individual kinetic constants being treated as a legitimate population of data points for statistical analysis.

RESULTS

Cytochrome P-450-dependent estrogen 2-hydroxylase activity in liver microsomes required NADPH cofactor, and activity was inhibited by SKF-525A (1 mM) as well as by carbon monoxide. The data in Table 1 show the effect of treatment of ovariectomized rats with several doses of ethinylestradiol (EE) on *in vitro* catechol estrogen formation. Estrogen 2-hydroxylase activity was increased significantly 39 and 100% above control levels in microsomes prepared from animals which had received 50 and 100 μg EE respectively. Thus, short-term administration of the synthetic estrogen ethinylestradiol appeared to elicit a dose-dependent increase in microsomal catechol estrogen formation.

In subsequent experiments, animals were treated with 100 μg EE, either alone or in combination with the antiestrogen tamoxifen (TAM), in an effort to evaluate the mechanism by which ethinylestradiol increased catechol estrogen formation. Administration of 100 μg daily doses of EE or TAM for 3 days resulted in significantly increased uterine weight, from 92 ± 9 mg (mean ± S.E.) in control animals to 254 ± 8 for EE- ($P < 0.01$), 141 ± 19 for TAM- ($P < 0.05$) and 217 ± 27 for EE + TAM- ($P < 0.01$) treated animals respectively. Data in Table 2 show the results of administration of EE, TAM, or EE + TAM on liver weight and the composition of hepatic microsomes. While total liver weight was not affected significantly by any treatment, the protein content of liver microsomes was increased 2-fold following administration of EE, TAM and EE + TAM ($P < 0.01$). Cytochrome P-450 content of liver microsomes did not change with EE or EE + TAM treatment, but it was increased 25% by TAM administered alone.

The effects of treatment of rats with EE, TAM, or EE + TAM on estrogen 2-hydroxylase activity in liver microsomes are shown in Fig. 1. Catechol estrogen formation *in vitro* was measured over a substrate concentration range of 0.5 to 50 μM, and enzyme activity was expressed as the turnover number, per nmole cytochrome P-450. Microsomes from EE-treated animals exhibited a 2-fold increase in enzyme activity at all substrate concentrations when compared to not only control, but also TAM and EE + TAM preparations. These differences were significant ($P < 0.05$) for all three groups at substrate concentrations between 0.5 and 10 μM when compared to the EE group. Thus, tamoxifen administered alone did not alter enzyme activity. Furthermore, microsomes from EE + TAM-treated animals did not exhibit the enhanced enzyme activity observed with EE administered alone.

Figure 2 shows double-reciprocal plots of enzyme

Table 1. Effects of administration of ethinylestradiol on estrogen 2-hydroxylase activity in liver microsomes*

Treatment		Estrogen 2-hydroxylase (nmoles/nmole P-450/min)
Control	(4)	0.46 ± 0.03
EE, 50 μg	(3)	0.64 ± 0.02†
EE, 100 μg	(5)	0.90 ± 0.16†

* Ovariectomized rats were treated with ethinylestradiol (EE) in doses of 50 or 100 μg/day for 3 days. Estrogen 2-hydroxylase activity was measured in liver microsomes at a 10 μM substrate concentration. Values are the means ± S.E. of the number of animals given in parentheses.

† $P < 0.05$ compared to control.

Table 2. Effects of administration of ethinylestradiol and tamoxifen on liver weight and cytochrome P-450 and protein content of hepatic microsomes*

Treatment		Liver wt (g)	Microsomes† (mg protein/g)	(nmoles P-450/g)
Control	(4)	10.0 ± 1.2	25.7 ± 1.7	6.1 ± 0.3
EE	(5)	9.7 ± 0.7	44.5 ± 4.4‡	6.4 ± 1.0
TAM	(4)	7.8 ± 0.5	53.5 ± 2.6‡	8.5 ± 0.8§
EE + TAM	(5)	7.9 ± 0.3	61.7 ± 4.6‡	7.1 ± 0.4

* Values are the mean ± S.E. of the number of animals given in parentheses.
† Content expressed per microsomes from 1 g liver.
‡ $P < 0.01$ vs control.
§ $P < 0.05$ vs control.

activity as a function of substrate concentration for microsomes from control and EE-treated animals. Reciprocal values for activity in EE microsomes were significantly different from control at all substrate concentrations. Analysis of variance of linear regression showed that the relationships were linear ($P < 0.001$) for both control and EE plots. The regression equations are indicated in the legend of Fig. 2 with regression coefficients (slopes) being significantly different between control and EE microsomes ($P < 0.001$). In data not shown, double-reciprocal plots of enzyme activity in microsomes from TAM- and EE + TAM-treated animals were also both linear with correlation coefficients of 0.98 ($P < 0.001$.). The regression equations were $y = 1.89 + 3.45x$ for TAM, and $y = 2.02 + 2.55x$ for EE + TAM respectively.

Data in Table 3 show the apparent kinetic constants obtained for estrogen 2-hydroxylase activity in microsomes from the four treatment groups. While apparent K_m values were virtually identical for enzyme activity in control and EE microsomes, apparent V_{max} values were 2-fold greater following estrogen treatment. Tamoxifen administered alone did not alter either kinetic constant. In microsomes from EE + TAM-treated animals, apparent V_{max} was identical with values for control and TAM preparations, and the apparent K_m was similar to that observed with TAM. Thus, ethinylestradiol administration was associated with a specific increase in the apparent V_{max} of estrogen 2-hydroxylase activity, and this effect was not observed when tamoxifen was co-administered with the estrogen.

DISCUSSION

The hepatic enzyme system involved in formation of 2-hydroxy estrogen metabolites in the rat has been demonstrated to be a cytochrome P-450-dependent monooxygenase [1, 2]. Studies on the hormonal regulation of this enzyme indicate that catechol estrogen formation is several-fold greater in liver microsomes from male rats compared to females [17, 18]. In this regard, estrogen 2-hydroxylase activity exhibits the typical pattern of sexual dimorphism described for microsomal steroid metabolism in the rat [7–9]. While 2-hydroxylation in males is androgen dependent, little is known about endo-

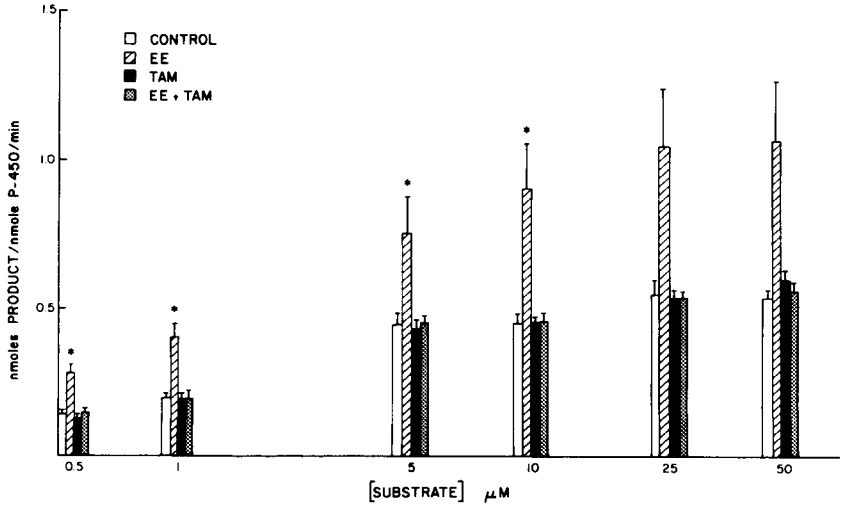

Fig. 1. Estrogen 2-hydroxylase activity in liver microsomes *in vitro* as a function of substrate concentration. Ovariectomized rats were treated with ethinylestradiol (EE) or tamoxifen (TAM) as described in the text. Values are the mean ± S.E. of four control, five EE-, four TAM- and five EE + TAM-treated animals. Key: (*) $P < 0.05$ compared to control, TAM, and EE + TAM at each substrate concentration.

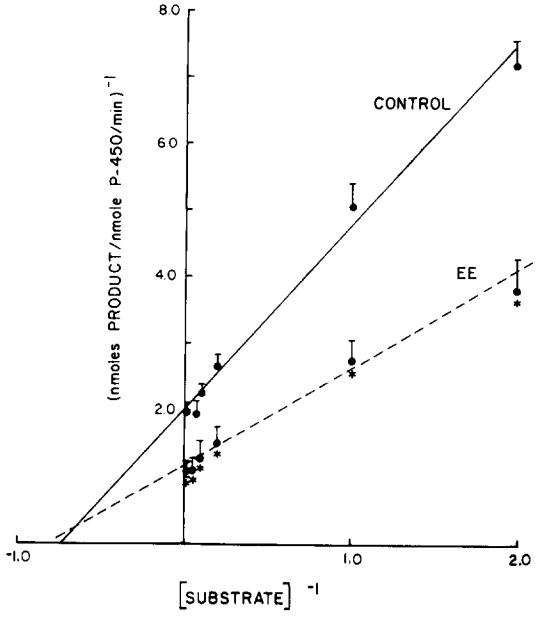

Fig. 2. Double-reciprocal plots of estrogen 2-hydroxylase activity as a function of substrate concentration (μM) in liver microsomes from control and ethinylestradiol (EE)-treated rats. Values are the mean ± S.E. of four control and five EE animals. Key: (*) $P < 0.05$. The correlation coefficients for both control and EE lines were 0.99 ($P < 0.01$) with the regression equation for control being $y = 1.98 + 2.68x$ and for EE $y = 1.14 + 1.38x$.

crine regulation of this enzyme in female rats where this pathway should play a significant role in the metabolism of endogenous and exogenous estrogens. In the present study, estrogen 2-hydroxylase activity was increased by administration of ethinylestradiol at doses which have been reported previously to suppress other steroid hydroxylase activities [7]. Considering the physiologic and pharmacologic importance of the estrogen 2-hydroxylation pathway, catechol estrogen formation activity might well be regulated by concentrations of available substrate [19].

The nonsteroidal antiestrogen tamoxifen is one of a class of compounds which prevents estrogens from exerting their full effects on some estrogen-responsive tissues. These compounds are of particular interest because they display a spectrum of activity which varies from pure antagonist, to partial antagonist, to full agonist, depending on animal species, target tissue and the particular response parameter under study [20–22]. In the present study, tamoxifen blocked the ethinylestradiol-associated stimulation of estrogen 2-hydroxylase activity, behaving as a pure antagonist. It should be noted, however, that tamoxifen itself is a substrate which undergoes hydroxylation in liver microsomes [23] and, thereby, could exert effects on microsomal catechol estrogen formation which involve both competitive and non-competitive mechanisms [24]. While tamoxifen treatment was found to increase cytochrome P-450 content of liver microsomes, no significant change was observed in either apparent K_m or V_{max} of estrogen 2-hydroxylase reaction. These data are consistent with other reports [24, 25] that tamoxifen administered to female rats has no effect on several drug metabolism enzyme activities. Thus, administration of ethinylestradiol in the present study appears to selectively increase apparent V_{max}, or the amount of cytochrome P-450 involved in catechol estrogen formation, an effect which is selectively blocked by co-administration of tamoxifen.

To fully understand the mechanisms involved in the regulation of hepatic microsomal enzymes, the sites of steroid action must be established. Most data support the concept that sex steroids act on the liver via an effect on the pituitary, and not via an effect on the liver itself [8]. Central to these data is the concept that a pituitary factor, feminotropin, actively suppresses steroid hydroxylase activities in female rat liver microsomes. Thus, high levels of steroid hydroxylase activities in male rats appear to reflect the absence of feminotropin. While none of the pituitary hormones studied to date can mimic pituitary transplantation in these effects, secretion seems to follow that of prolactin and/or growth hormone in a number of instances [8]. As defined by Gustafsson and his co-workers for sexually dimorphic steroid hydroxylase enzymes, the EE-associated increase in estrogen 2-hydroxylase observed in the present study would be consistent with less feminotropin action, i.e. masculinization, an interpretation which seems paradoxical.

Alternatively, although most data indicate that estrogens act on liver microsomal enzymes via the pituitary, studies to date have not excluded a role for direct action on liver estrogen receptors. Several laboratories have demonstrated the presence of estrogen receptors in liver cytosol from female rats [26, 27]. Ethinylestradiol is much more potent than estradiol in promoting translocation of the hepatic receptor [28]. In the present study, although estrogen

Table 3. Apparent kinetic constants of estrogen 2-hydroxylase activity in liver microsomes*

Treatment		Apparent K_m (μM)	Apparent V_{max} (nmoles/nmole P-450/min)
Control	(4)	1.36 ± 0.04	0.52 ± 0.04
EE	(5)	1.33 ± 0.20	1.01 ± 0.19†
TAM	(4)	1.79 ± 0.33	0.53 ± 0.02
EE + TAM	(5)	1.88 ± 0.44	0.56 ± 0.06

* Values are means ± S.E. of the number of animals given in parentheses.
† $P < 0.05$.

2-hydroxylase activity was enhanced significantly following the 50 μg dose of EE, the V_{max} increase was observed only with the higher 100 μg dose of synthetic estrogen (unpublished observation). The high dose requirement observed in the present study would be consistent with that required for translocation of the liver estrogen receptor system [28]. In other studies, a 100 μg dose of tamoxifen has been reported to elicit regression of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary tumors [20], as well as to be a potent agonist in rat liver as determined by elevation of plasma renin substrate levels [21]. In the green monkey [22], other antiestrogens have been shown to act as estrogen agonists in increasing plasma cortisol-binding globulin (CBG) and decreasing haptoglobin levels, yet also to act as antagonists in preventing estrogen-stimulated increases in T_4 binding globulin.

In summary, ethinylestradiol appears to enhance its own metabolism to a catechol product, and tamoxifen behaves as an antagonist in blocking this effect. While these data implicate an estrogen receptor in the regulation of estrogen 2-hydroxylase activity, it is not known whether the response is mediated by direct action on the liver or via the pituitary through feminotropin. The former mechanism would imply that a specific steroid hydroxylase activity like estrogen 2-hydroxylase activity may be under multi-hormonal control, i.e. presumably suppressed by feminotropin, but enhanced by ethinylestradiol. Such multihormonal control by peptide and steroid factors has been demonstrated previously for renal vitamin D 1α-hydroxylase activity [29]. These data may help to elucidate the effects of contraceptive steroids on liver function.

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