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MESTRANOL-INDUCED HYPERTENSION: CHARACTERIZATION OF CYTOCHROME P-450 DEPENDENT CATECHOL ESTROGEN FORMATION IN BRAIN MICROSOMES*

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Abstract—An animal model of estrogen-induced hypertension was used to study the effects of chronic administration of the synthetic estrogen mestranol on cytochrome P-450 content and catechol estrogen formation in brain microsomes. Cytochrome P-450 content of brain microsomes from untreated female rats in estrus was 0.034 nmole/mg protein and the dithionite-reduced carbon monoxide absorbance peak (λ_{max}) was 452 nm. Catechol estrogen formation in brain microsomes was optimal in the presence of both NADPH and NADH cofactors with an apparent K_m value of 71 μM for 17β-estradiol substrate. Brain microsomes from animals in estrus and diestrus were compared, and no significant differences were observed in cytochrome P-450 content, or in the apparent K_m and V_{max} values of catechol estrogen formation. Administration of mestranol, 15 μg biweekly, for 3–4 weeks resulted in a significant increase in systolic blood pressure in unanesthetized female rats. Mestranol treatment was not associated with a change in microsomal cytochrome P-450 content or the spectral λ_{max}. At 10 μM substrate concentration, catechol estrogen formation in microsomes from mestranol-treated animals was increased 2- to 3-fold, with enzyme activity being expressed either per mg protein or per nmole cytochrome P-450. In contrast, no difference was observed between groups when enzyme activity was measured at 100 μM substrate concentration. These data suggest that chronic administration of a synthetic estrogen can regulate the enzyme system involved in formation of brain catechol estrogen metabolites, a mechanism which may alter the biological impact of the parent steroid.

The estrogen component of contraceptive steroids has been most associated with the increased risk of hypertension for oral contraceptive users [1, 2]. Steroid contraceptive drugs have been reported to affect catecholamine concentrations and turnover in discrete areas of the brain [3–6]. While most steroid-induced changes in neurotransmission have been related to gonadotrophin secretion [7], estrogen–catecholamine interactions may well affect central regulation of blood pressure by noradrenergic neurons. In studies with an animal model of hypertension, Lew [8, 9] reported that chronic administration of mestranol, a synthetic estrogen, resulted in a significant elevation of systolic blood pressure. Furthermore, the extent of mestranol-induced hypertension was correlated with depletion of norepinephrine content in the hypothalamus.

Recent evidence suggests that estrogens may influence neuronal function by non-receptor mechanisms which involve metabolism of estrogens to catechol products. The hypothalamus and other brain tissues are capable of converting natural and synthetic estrogens to 2- and 4-hydroxylated catechol metabolites [10–13]. The catechol estrogen forming enzyme has been characterized as a microsomal cytochrome P-450 dependent monooxygenase [12]. Based upon their catechol structure, the 2-hydroxy estrogens have been shown to be potent competitive inhibitors of both tyrosine hydroxylase and catechol-*O*-methyl transferase activities in a manner similar to catecholamines [14–16]. Thus, this unique steroid metabolite may be part of the biochemical mechanism by which gonadal steroids influence catecholamines in the central nervous system.

The present study was initiated to evaluate whether chronic administration of a pharmacologic dose of the synthetic estrogen mestranol regulates the activity of enzymes which catalyze formation of catechol estrogens in the brain, an effect which could be associated with prolonged effects on catecholamines. The present study characterizes cytochrome P-450 content and catechol estrogen formation activity of brain microsomes from female rats which exhibit hypertension following mestranol treatment.

MATERIALS AND METHODS

Chemicals. [^{3}H]-*S*-adenosyl-*l*-methionine (68 Ci/mmole) was purchased from the New England Nuclear Corp. (Boston, MA). Mestranol (ethinylestradiol 3-methyl ether) was obtained from Steraloids (Wilton, NH). The following were purchased from the Sigma Chemical Co. (St. Louis, MO): 17β-estradiol, *S*-adenosyl-*l*-methionine, NADPH, NADH, ascorbic acid and catechol-*O*-methyl transferase.

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Animal treatment. Adult female Sprague–Dawley rats were housed in groups of three in wire cages with water and Purina Laboratory Chow *ad lib*. The room was maintained on a 7:00 a.m. to 7:00 p.m. light cycle. Animals received mestranol biweekly, i.p., 15 μg in 1 ml of 2% ethanol in 0.85% saline or vehicle alone. Systolic blood pressure of unanesthetized animals was measured indirectly from the tail by the microphonic manometer technique [17] using a transducer-monitor coupler (E & M Instruments, Inc.) connected to a Sanborn Two Visopolygraph. This indirect measurement has been reported to correlate well with the direct measurement of systolic blood pressure obtained by arterial cannulation. Blood pressure was measured on two separate occasions prior to treatment, in order to establish basal values, and then weekly during the experiment. Five separate recordings were averaged on each occasion.

Brain microsomes. After 4 weeks of treatment animals were decapitated, and the whole brain, excluding the pituitary, was rapidly excised. Brain microsomes were prepared by a modification of the method of Marietta *et al.* [18] which was developed to optimize cytochrome P-450 recovery and stability. All steps were carried out at 0–4°. The brain was rinsed three times in ice-cold 0.1 M potassium phosphate buffer, pH 7.4, and then homogenized, 1 g/5 ml buffer, with a motor-driven pestle of a glass tissue homogenizer. The preparation was sonicated for 15 sec and then centrifuged for 10 min at 1000 *g*. The supernatant fraction was centrifuged at 9000 *g* for 20 min, and then at 105,000 *g* for 60 min, with the microsomal pellet being resuspended in 0.1 M potassium phosphate buffer, pH 7.4, so that 1 ml contained microsomes from 1 g of brain. Cytochrome P-450 was measured by the dithionite-reduced carbon monoxide (CO)-difference spectrum according to Omura and Sato [19]. Protein concentration was determined by the method of Lowry *et al.* [20].

Enzyme activity. Catechol estrogen formation was measured by a modification of the radioenzymatic method of Paul *et al.* [12]. The incubation mixture contained 0.1 M potassium phosphate buffer, pH 7.4, 5 mM $MgCl_2$, 0.5 mM ascorbic acid, 0.6 mM NADPH, 0.6 mM NADH, 2 units catechol-*O*-methyl transferase and [^{3}H]-*S*-adenosyl methionine (1 μCi/15.1 pmoles). 17β-Estradiol substrate was present in concentrations between 4 and 420 μM (added in 0.002 ml ethanol), and 0.25 to 0.35 mg of microsomal protein was added in a final volume of 0.110 ml. Blanks consisted of enzyme without NADPH/NADH cofactors. Product was extracted and characterized by thin-layer chromatography as described by Paul *et al.* [12].

Statistical analysis of the data was by Student's *t*-test.

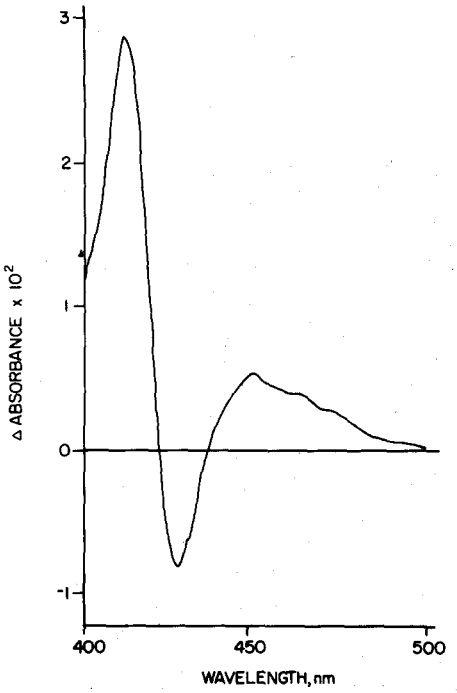

Fig. 1. Carbon monoxide (CO)-difference spectrum of dithionite-reduced brain microsomes. Microsomes (1.07 mg protein in 1 ml of 0.10 M KPO_4, pH 7.4) were mixed with dithionite and added to each cuvette. The base line was adjusted, CO was added to the sample only, and the difference spectrum was recorded.

RESULTS

Cytochrome P-450 and catechol estrogen formation in brain microsomes. A method for the preparation of brain microsomes was developed to optimize cytochrome P-450 recovery and stability. Figure 1

Table 1. Effects of incubation conditions on the catechol estrogen formation activity of brain microsomes*

Conditions	Activity: pmoles·(mg protein)$^{-1}$·min^{-1}	Activity: % Control
NADPH + NADH	0.107 ± 0.005	100
NADPH + O_2	0.092 ± 0.005	88
NADPH (0.6 mM)	0.071 ± 0.009	66
NADH (0.6 mM)	0.047 ± 0.002	43
Minus cofactors	0.032 ± 0.003	31
Minus estradiol	0.014 ± 0.001	13

* 17β-Estradiol substrate concentration was 100 μM. Each value is the mean ± S.E. of four microsomal preparations from brain tissue (excluding pituitary) of adult female rats.

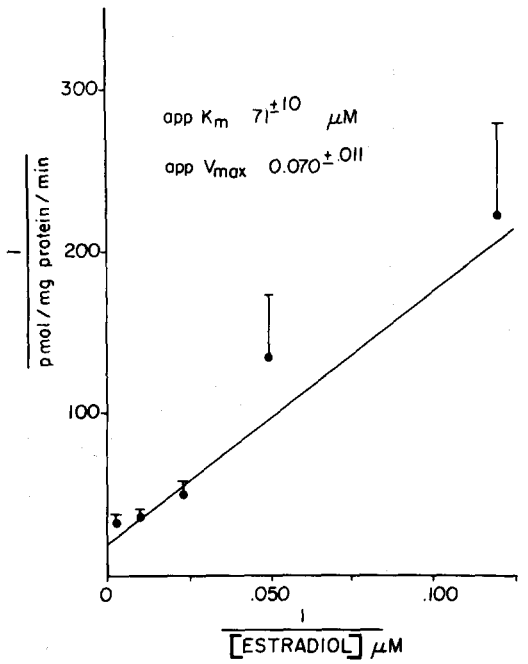

Fig. 2. Lineweaver–Burk kinetics for catechol estrogen formation activity in brain microsomes from female rats. Values are the means ± S.E. of five animals. The correlation coefficient for the relationship was $r = 0.98$, $P < 0.001$.

shows a typical dithionite-reduced CO-difference spectrum of brain microsomes from untreated animals. The absorbance maximum at 452 nm reflects the absorbance difference between the CO complex of reduced cytochrome P-450 and the reduced cytochrome alone. The other absorbance maximum at 418 nm was not due to cytochrome b_5 or hemoglobin (CO bubbled into the reference cuvette did not reduce the peak) and presumably represents inactive cytochrome P-420. This spectrum agrees closely with that reported by Cohn *et al.* [21] for brain microsomes. The relative sizes of the 450 and 420 nm peaks were not altered by the presence of EDTA in the buffer to prevent lipid peroxidation. Sonication proved to be the step most essential to maintain stable cytochrome P-450 content and to minimize the relative amount of material absorbing at 420 nm. Sonication also increased the protein content of the microsomal preparation by 4- to 5-fold. Microsomes from untreated rats in estrus contained 0.034 ± 0.007 nmole cytochrome P-450/mg protein ($\bar{X}$ ± S.E.), and these values are in close agreement with those reported by Sasame *et al.* [22] and Marietta *et al.* [18].

Catechol estrogen formation in brain microsomes from untreated animals was linear for 30 min in the presence of 0.05 to 0.35 mg protein. A study of cofactor requirements indicated that enzyme activity was optimal in the presence of both NADPH and NADH (Table 1). It was consistently observed that activity in the absence of these two cofactors was 2- to 4-fold higher than activity in the absence of 17β-estradiol substrate alone. This NADPH/NADH independent formation of catechol estrogens was observed in brain, but not in liver microsomes. Substrate kinetics for catechol estrogen formation are shown in Fig. 2. Saturation curves were obtained by varying the amount of substrate over a concentration range of 4.2 to 420 μM. Enzyme activity in the absence of NADPH/NADH was used as the appropriate blank at each estradiol concentration because the cofactor-independent catechol estrogen formation increased as a function of substrate concentration. The apparent K_m value was 71 μM, and the apparent V_{max} was 0.070 pmoles per mg protein per min for enzyme activity in brain microsomes from female rats in estrus. Data in Table 2 compare brain microsomes prepared from cycling rats in estrus and diestrus. Linear double-reciprocal plots were obtained for all microsomal preparations, and no significant differences were observed in either cytochrome P-450 content or the apparent K_m and apparent V_{max} values for catechol estrogen formation during these two stages of the reproductive cycle.

Effects of mestranol on systolic blood pressure. Chronic mestranol treatment was associated with a significant 11–16 mm Hg increase in systolic blood pressure in unanesthetized rats (Table 3). This elevation in blood pressure was observed after treatment for 3 ($P < 0.001$) and 4 weeks ($P < 0.01$) when compared to both control animals and pretreatment levels.

Effects of mestranol treatment on brain microsomes. Data in Table 4 show the composition of brain microsomes from mestranol-treated animals. No differences were observed between control and treated animals in body or brain weights. Brain microsomes from the two groups were comparable in protein and cytochrome P-450 content, as well as the λ_{max} of the reduced CO-difference spectrum.

Table 2. Cytochrome P-450 content and catechol estrogen formation in brain microsomes from female rats in estrus and diestrus*

	Cytochrome P-450 (nmoles/mg protein)	Catechol estrogen formation	
		app K_m†	app V_{max}‡
Estrus (5)	0.034 ± 0.007	70 ± 11	0.070 ± 0.011
Diestrus (6)	0.038 ± 0.014	40 ± 9	0.050 ± 0.12

* Estrus and diestrus were determined by vaginal cytology at 9:00 a.m. The apparent kinetic constants, K_m and V_{max}, were determined for individual microsomal preparations. Each value is the mean ± S.E. of the number of animals given in parentheses.

† The unit for K_m is μM.

‡ Expressed as pmoles product per mg protein per min.

Table 3. Effect of mestranol treatment on systolic blood pressure in female rats*

	Systolic blood pressure (mm Hg)		
	Pretreatment	Post-treatment	
		3 Weeks	4 Weeks
Control	113 ± 4	111 ± 2	111 ± 4
Mestranol	116 ± 3	127 ± 2†‡	127 ± 2‡§

* Animals were treated with mestranol, 15 μg i.p. biweekly. Values are means ± S.E. of five animals in each group.
† $P < 0.001$, compared to control.
‡ $P < 0.05$, compared to pretreatment.
§ $P < 0.01$, compared to control.

Catechol estrogen formation activity in brain microsomes was assayed at two substrate concentrations (Fig. 3). No difference was observed between microsomal preparations when enzyme activity was measured at a concentration of 100 μM estradiol. In contrast, catechol estrogen formation measured at low substrate concentration (10 μM) was increased 2-fold in microsomes from mestranol-treated animals ($P < 0.05$). When product formation is expressed as the turnover number, per nmole cytochrome P-450, catechol estrogen formation in treated microsomes was increased 3-fold at 10 μM estradiol ($P < 0.05$) and activity was enhanced, but not significantly, at the high substrate concentration. There was no correlation between cytochrome P-450 content and the level of microsomal enzyme activity.

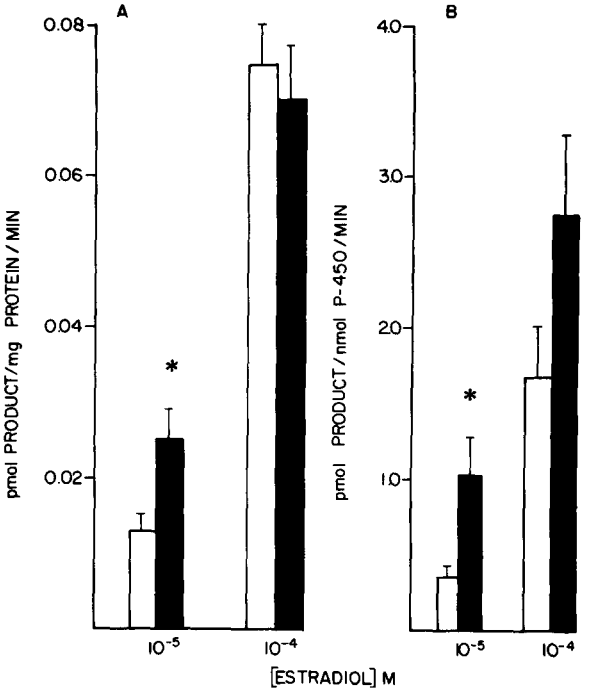

Fig. 3. Catechol estrogen formation activity of brain microsomes from untreated and mestranol-treated female rats. Enzyme activity was measured at two different substrate concentrations, 10 and 100 μM 17β-estradiol. Values are the means ± S.E. of four control (open bars) and five mestranol-treated (closed bars) animals. Key: (*) $P < 0.05$.

DISCUSSION

The catechol estrogen forming activities in brain [12], as well as liver [23], have been characterized as cytochrome P-450 dependent monooxygenase enzymes. Other studies have demonstrated the presence of cytochrome P-450 in rat brain microsomes [18, 21, 22], as well as the catalytic activity of these enzymes in the metabolism of several drugs and xenobiotics [18, 21, 24–26]. Brain cytochrome P-450 dependent enzymes differ quantitatively and, in some aspects, qualitatively from those in the liver [18, 27]. Thus, cytochrome P-450 content in brain microsomes is 10–15% of that present in liver microsomes, while the absorbance maximum (λ_{max}) of the reduced CO-difference spectrum of brain cytochrome is 452 and not 450 nm. The apparent K_m for catechol estrogen formation reported in the present study for female rats is comparable to that reported by Hoffman *et al.* [27] for male rats. It is noteworthy that we consistently observed an appreciable level of catechol estrogen formation in the absence of NADPH and NADH. The cofactor-independent enzyme activity varied as a function of substrate concentration and was characteristic of brain, but not of liver microsomes. The data suggest the involvement of an alternative non-P-450 mediated

Table 4. Effect of mestranol treatment on brain weight and microsomal cytochrome P-450 and protein content*

	Body wt (g)	Brain wt (g)	Microsomes		
			(mg protein/ g brain)	(nmoles P-450/ mg protein)	λ_{max}† (nm)
Control	229 ± 16	1.93 ± 0.07	5.35 ± 0.28	0.053 ± 0.014	452
Mestranol	230 ± 4	1.87 ± 0.04	5.72 ± 0.37	0.030 ± 0.007	452

* Values are means ± S.E. of five animals in each group. Cytochrome P-450 was calculated using a molar extinction coefficient of 91 $nM^{-1}\,cm^{-1}$.
† λ_{max} of reduced CO-difference spectrum.

mechanism in brain catechol estrogen formation which warrants further investigation.

The physiologic significance of catechol estrogen formation in brain microsomes depends on several properties of the cytochrome in this tissue and its hormonal regulation. Both hypothalamic and pituitary tissue contain the greatest concentration of endogenous catechol estrogens [28], and these tissues show the highest activity for formation of these products *in vitro* [10, 29, 30]. These findings led to the proposal that catechol estrogen formation *in situ* may be of biological significance in the hypothalamic regulation of pituitary hormone secretion. In this context, estrogen 2-hydroxylase activity was suppressed by opiates in a manner analogous to the effects of opiates on LH secretion in male rats [31], while in other experiments estrogen 2-hydroxylase activity increased greatly at proestrus in female rats [32]. In the present study, brain microsomes from animals in estrus and diestrus were compared, and no differences were observed in either cytochrome P-450 content or in the apparent K_m and V_{max} values for catechol estrogen formation. It must be emphasized, however, that the radioenzymatic assay does not distinguish between formation of 2- and 4-hydroxyestradiol products, two catechol estrogens which have been reported to exert markedly different effects on the induction of ovulation [33].

The pharmacologic significance of catechol estrogen formation in brain microsomes would depend on the mechanisms by which exogenous estrogens may influence their own disposition and biological impact. The objective of the present study was to determine whether a synthetic estrogen can act as a regulator of the enzyme system involved in formation of catechol estrogen metabolites in the brain. Chronic administration of mestranol was not associated with a change in microsomal cytochrome P-450 levels or spectral characteristics. Catechol estrogen formation activity in microsomes from mestranol-treated animals was increased significantly at low, but not at high substrate concentrations. These data would be consistent with a change in the affinity of the brain enzyme system for an estrogen substrate following mestranol treatment, evidence which suggests that this synthetic estrogen can enhance its own metabolism to catechol products.

The present study confirms the report of Lew [8] that chronic administration of mestranol to female rats resulted in a significant elevation of systolic blood pressure. There is considerable evidence that ovarian hormones have direct effects on central catecholamine levels or turnover [3, 5, 6]. Catechol estrogens but not parent estrogens or methylated catechol metabolites, competitively inhibit the activities of tyrosine hydroxylase and catechol-*O*-methyl transferase enzymes *in vitro* in the same manner as catecholamines [14–16]. Administration of estrogens to ovariectomized rats was reported to selectively depress tyrosine hydroxylase activity in the hypothalamus where dopaminergic neurons concentrate estrogen [34]. While the central effects of estrogen have been primarily related to gonadotrophin secretion [7], Lew has reported an association between mestranol-induced hypertension and depletion of hypothalamic norepinephrine content [8, 9]. Pharmacologic studies have related norepinephrine concentration in the hypothalamus to the regulation of blood pressure [35]. Reports of these central effects thus stress the importance of pursuing studies to clarify the significance of steroid-induced monoamine changes for both their contraceptive action and their side effects. Studies are in progress to evaluate the relationship between catechol estrogen metabolites and altered monoamine concentrations in this model of estrogen-induced hypertension.

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