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ESTROGEN-2-HYDROXYLASE IN THE RAT

DISTRIBUTION AND RESPONSE TO HORMONAL MANIPULATION

ANDREW R. HOFFMAN,*† STEVEN M. PAUL*‡ and JULIUS AXELROD
Section on Pharmacology, Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, MD 20014, U.S.A.

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Abstract—Estrogen-2-hydroxylase activity in various rat tissues was surveyed. While the liver, followed by brain, had the highest enzyme activity, nearly all tissues investigated in the male rat possessed 2-hydroxylase activity. Neither the ovary nor the virgin uterus, however, could synthesize 2-hydroxyestrogens *in vitro*. Enzyme activities in the male brain and liver were higher than in corresponding female tissues. In the male rat, cytochrome P-450 inducers did not alter brain estrogen-2-hydroxylase activity, but castration caused a fall in enzyme activity in both the brain and liver. Liver estrogen-2-hydroxylase activity was decreased in both hyperthyroid and hypothyroid rats.

The catecholestrogens have been shown recently to be major metabolites of estrogen, both in laboratory animals and in humans [1]. Although their precise physiologic role is not known, catecholestrogens have been detected in brain, pituitary, liver and ovary [2], and have been shown to exhibit both estrogenic and antiestrogenic properties [1]. While assays for blood and urine catecholestrogens have been complicated by the instability of the compound, there have been reports of elevated 2-hydroxyestrogen levels in urine in thyroid disease [3] and in anorexia nervosa [4], a psychosomatic disorder accompanied by altered neuroendocrine homeostasis. The formation of 2-hydroxyestrone and 2-hydroxyestradiol, the principal catecholestrogens, is catalyzed by estrogen-2-hydroxylase, a microsomal cytochrome P-450-dependent mono-oxygenase [5–7]. In this report, we examine the tissue distribution of estrogen-2-hydroxylase in the rat and describe the effects of cytochrome P-450 enzyme inducers and various hormones on the activity of the enzyme. This study was made possible by an exceedingly sensitive radioenzymatic assay for the estrogen-2-hydroxylase [6].

MATERIALS AND METHODS

Chemicals

Estradiol-17β, testosterone, 3-methylcholanthrene, phenobarbital, nicotinamide adenine dinucleotide phosphate reduced form (NADPH), catechol-*o*-methyltransferase (COMT) (chromatographically pure), 2-methoxyesterone and 2-methoxyestradiol were purchased from the Sigma Chemical Co., St. Louis, MO. Dithiothreitol was obtained from Bethesda Research Laboratories, Inc., Rockville, MD. L-Thyroxine was purchased from Nutritional Biochemicals, Cleveland, OH. L-Ascorbic acid and *n*-heptane were from the Fisher Scientific Co., Fair Lawn, NJ. [^{3}H]*S*-adenosylmethionine (sp. act. 11.2 Ci/mmole) was purchased from the New England Nuclear Corp., Cambridge, MA. Tris base was purchased from Schwartz/Mann, Orangeburg, NY.

Preparation of COMT

COMT was purified from rat liver according to the procedure of Axelrod and Tomchick [8] as modified by Nikodejevic *et al.* [9]. Rat livers were homogenized in 4 vol. (w/v) of isotonic KCl in a glass homogenizer equipped with a loose-fitting motor-driven Teflon pestle. The homogenate was filtered through cheesecloth and centrifuged at 14,000 *g* for 30 min. The supernatant fraction was then centrifuged for 60 min at 105,000 *g*. After filtering through glass wool to remove fat particles, the high speed superantant fraction was adjusted to pH 5.3 with 1 M acetic acid. The suspension was centrifuged at 14,000 *g*, and the pH of the resulting supernatant fraction was readjusted to pH 7.0. Fractionation and backwash with ammonium sulfate was carried out according to the procedure of Nikodejevic *et al.* [9]. The final precipitate was dissolved in 40 ml of 0.001 M phosphate buffer (pH 7.0) and dialyzed overnight against three changes of 0.01 M phosphate buffer (pH 7.0) containing 0.001 M dithiothreitol. Following dialysis the enzyme preparation was re-centrifuged for 2 hr at 105,000 *g* to remove a yellow-brown precipitate. The enzyme preparation was stored at −20° with no loss of activity for at least 1 year.

Chromatographically pure enzyme, obtained commercially, was found to be unstable and of variable activity. Therefore, all assays were performed with enzyme prepared in our laboratory. In contrast to

* Research Associate in the Pharmacology-Toxicology Program, National Institute of General Medical Sciences, National Institutes of Health, Bethesda, MD 20014.
† Address reprint requests to: Andrew R. Hoffman, Laboratory of Clinical Science, National Institute of Mental Health, Bldg. 10, Rm. 2D-47, Bethesda, MD 20014.
‡ Present address: Clinical Psychobiology Branch, National Institute of Mental Health, Bethesda, MD 20014.

a previous report [10], no detectable estrogen-2-hydroxylase activity was present in this soluble enzyme preparation.

Preparation of tissues

Adult male and female Sprague–Dawley rats (150–250 g, Zivic–Miller Laboratories, Allison Park, PA) were housed under diurnal lighting conditions with free access to food and water. In the experiments with 3-methylcholanthrene and phenobarbital, 80–100 g male rats were used. Rats were acclimated to the NIH animal room for at least 1 week and were decapitated. Tissues were removed rapidly and then homogenized in 4 vol. of ice-cold isotonic sucrose or KCl. Entire fetuses from female rats who were 17 days pregnant were homogenized in the same manner as other tissues. The mitochondrial, microsomal and soluble fractions were prepared by differential centrifugation, and the particulate fractions were resuspended in 10 mM Tris–HCl buffer (pH 7.4). All assays were performed the same day that the animals were killed.

Enzyme assay

Estrogen-2-hydroxylase was assayed by the method of Paul *et al.* [6] in which the hydroxylation of estradiol is coupled with rapid *O*-methylation by COMT. The specificity of this method has been established by subjecting the reaction products to thin-layer chromatography, direct probe mass spectrometry [6], and recrystallization to constant specific activity [10]. In the present study, the incubation mixture consisted of 50 μl of 10 mM Tris–HCl buffer (pH 7.4), 10 μl of 1 M $MgCl_2$, 5 μl of [^{3}H]*S*-adenosylmethionine (sp. act. 11.2 Ci/mmole), 50 μg of partially purified COMT, 5 μl of 10 mM L-ascorbic acid, substrate consisting of 50 nmoles of estradiol-17β in 2 μl absolute ethanol, 0.06 μmole NADPH, and microsomal protein in a total volume of 150 μl. Reactions were started by placing the tubes in a shaking water bath at 37°. A portion of the enzyme preparation was analyzed for protein concentration by the method of Lowry *et al.* [11]. Blanks consisted of heat-treated enzyme including substrate and enzyme without substrate, and were not significantly different from each other. After incubation for 10 min at 37°, the reaction was stopped by the addition of 0.5 ml of 0.5 M borate buffer (pH 10.0). The radioactive *O*-methylated catechol estrogens were extracted into 6 ml of *n*-heptane by shaking vigorously for 30 sec, followed by separation of the organic phase by centrifugation. Two ml portions of the organic phase were transferred to scintillation vials, evaporated to dryness in a chromatography oven (80°), and counted for radioactivity directly in 10 ml Aquasol (New England Nuclear Corp., Cambridge, MA).

Product identification was confirmed in this study by subjecting the remaining organic phase containing the reaction products (approximately 3.5 to 4.0 ml reduced by evaporation under N_2) to thin-layer chromatography (t.l.c.) in two different solvent systems. Authentic 2-methoxyestrone and 2-methoxyestradiol were added as nonradioactive carriers. Thin-layer chromatography was performed on silica gel 125 mm 60 F-254 precoated plates (E. Merck, Germany). The solvent systems used were chloroform–methanol–acetic acid (96:3:1), and benzene–ethanol (9:1). Following development and drying of the plates, 1 cm sections were scraped individually into 12 ml conical glass-stoppered centrifuge tubes and 3 ml of absolute ethanol were added. After vortexing for 30 sec, the tubes were centrifuged and the clear ethanolic supernatant fractions were transferred to scintillation counting vials containing 10 ml Aquasol.

Thyroxine assay

Serum thyroxine levels were determined by radioimmunoassay [12].

Drug and surgical treatments

3-Methylcholanthrene (3-MC) and phenobarbital (PB) treatment. Young male rats were injected subcutaneously with 3-MC (20 mg/kg) in sesame oil once each day for 2 days or with PB (50 mg/kg) in propylene glycol twice daily for 5 days. Each experimental group also received injections of the other vehicle. Control animals were injected with both vehicles.

Castration. Adult male rats were castrated or subjected to sham-operation under ether anesthesia at Zivic–Miller Laboratories. They were killed a minimum of 4 weeks after surgery, at which time verification of orchiectomy was made.

Thyroidectomized animals. Adult male rats underwent thyroparathyroidectomy at the laboratory of the breeder. At the same operation, the parathyroid glands were autotransplanted into contiguous neck muscles. Hypothyroid and sham-operated animals were killed 4–6 weeks after surgery. Severe hypothyroidism was confirmed by the absence of detectable serum thyroxine at the time of death.

Hormone treatment. Testosterone (2 mg in 0.25 ml sesame oil) was injected subcutaneously once daily for 14 days, and the animals were killed on the following day. Thyroidectomized animals were made euthyroid by daily subcutaneous injections of 5 μg L-thyroxine [dissolved in methanol–ammonium hydroxide (3:1) and then diluted with 0.9% NaCl] in 0.10 ml vehicle. Hyperthyroidism was induced by injecting 100–500 μg L-thyroxine in 0.10 ml vehicle subcutaneously for 7–10 days. Serum T_4 levels on the 500 μg/day regimen were $7.8 + 0.3$ μg/dl, significantly higher than the control values (3.1 ± 0.2 μg/dl). All control animals received injections of vehicle alone.

RESULTS

Tissue distribution of estrogen-2-hydroxylase

Several endocrine and nonendocrine tissues were examined for estrogen-2-hydroxylase activity (Table 1). Although the liver had far more activity than any other organ, nearly every tissue studied had detectable enzyme activity. After the liver, the brain had the highest specific activity, followed by the kidney, testis, adrenal and lung. Measurable amounts of enzyme were also present in the pituitary, heart, placenta and fetus, but no activity could be detected in the uterus, an estrogen target tissue, or in the ovary, the major site of estrogen production. The

Table 1. Distribution of estrogen-2-hydroxylase in 8-* to 10-week-old rats

Organ	Activity* (± S.E.M.) Male	Female
Liver	9900 ± 300	1500 ± 200†
Brain	15.1 ± 1.7	8.7 ± 1.6‡
Kidney	8.1 ± 1.0	
Testis	5.7 ± 0.1	
Adrenal§	4.2 ± 0.8	
Lung	2.7 ± 0.6	
Pituitary‖	0.7 ± 0.1	
Heart	0.5 ± 0.3	
Placenta (17 days)		0.5 ± 0.04
Uterus		¶
Ovary		¶
Pineal	¶	
Fetus (17 days)		0.6 ± 0.04

* Activity is expressed in pmoles of 2-methoxyestradiol formed/mg of microsomal protein/10min incubation. N = 4.
† $P < 0.001$, different from male.
‡ $P < 0.05$, different from male.
§ Activity of mitochondrial protein was undetectable.
‖ Activity is expressed in mg of soluble protein.
¶ Activity was undetectable.

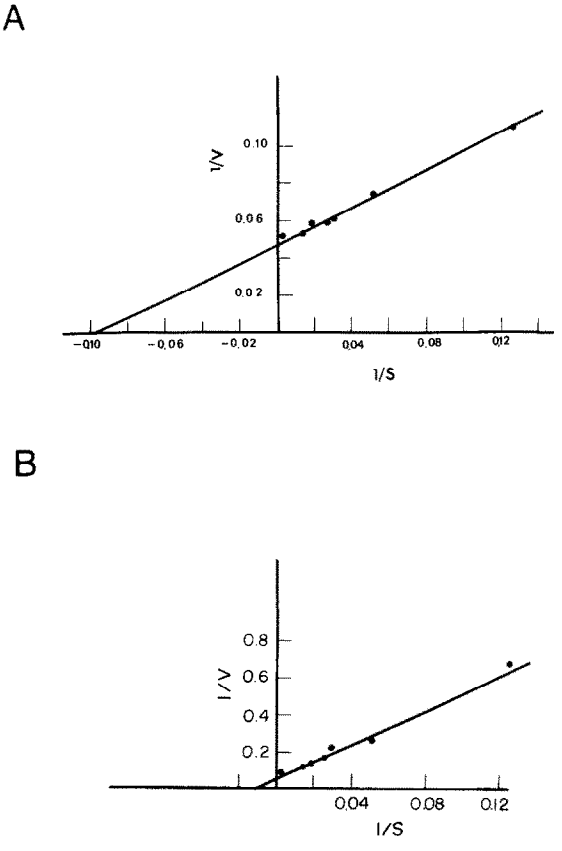

Fig. 1. Lineweaver–Burk kinetics for liver (A) and brain (B) estrogen-2-hydroxylase. The K_m for liver is 11 μM and for brain, 95 μM. V = pmoles of product formed/mg protein/10 min for brain (B). S = μM estradiol-17β.

estrogen-2-hydroxylase activity in the male brain and liver were higher than in the corresponding female tissues (Table 1).

Since cytochrome P-450-dependent mono-oxygenases in the adrenal gland are found in the mitochondria as well as in the microsomes [13], the subcellular distribution of estrogen-2-hydroxylase was examined in this tissue. No detectable enzyme activity was found in the crude mitochondrial or soluble fractions. All of the activity in the adrenal gland was found in the microsomal fraction.

Substrate kinetics for estrogen-2-hydroxylase in brain and liver were studied using estradiol-17β as substrate. The apparent K_m values (Fig. 1) of the brain (95 μM) and the liver (11 μM) enzymes differ by nearly one order of magnitude, suggesting that the enzymes responsible for estrogen-2-hydroxylation in these organs have different properties.

Effects of enzyme inducers

The effects of the microsomal enzyme inducers phenobarbital and 3-methylcholanthrene on the newly described cytochrome P-450-dependent enzyme in the brain [6] were examined. There was no change in brain enzyme activity after treatment with either phenobarbital or 3-methylcholanthrene (Table 2). Liver enzyme activity was also unchanged after phenobarbital [14] or 3-methylcholanthrene (data not shown).

Effects of hormones

The effects of various hormones upon the enzyme activity in the male brain and liver were studied next. High doses of testosterone had no effect on the enzyme activity, but castration resulted in significant decreases of enzyme activity in both brain and liver in male rats (Table 3). In both the hyperthyroid and

Table 2. Effect of P-450 inducers on brain estrogen-2-hydroxylase activity in the male rat*

Drug	Activity (± S.E.M.)
Control	3.5 ± 0.2
3-Methylcholanthrene	3.7 ± 0.2†
Phenobarbital	3.7 ± 0.2†

* Activity is expressed in pmoles of 2-methoxyestradial formed/mg of microsomal protein/10-min incubation. N = 8.
† Not significantly different from control value.

Table 3. Effects of sex hormones on brain and liver estrogen-2-hydroxylase activity in the male rat*

Hormonal manipulation	N	Brain	Liver
Control	13	15.6 ± 2.2	7700 ± 900
Castrated	13	8.8 ± 0.8†	3100 ± 400‡
Control	5	14.0 ± 2.3	7400 ± 800
Testosterone	5	13.9 ± 1.0	5140 ± 700

* Activity is expressed in pmoles of 2-methoxyestradiol formed/mg of microsomal protein/10-min incubation.
† $P < 0.01$, different from control.
‡ $P < 0.001$, different from control.

Table 4. Thyroid hormone and estrogen-2-hydroxylase activity in the male rat*

Thyroid status	N	Brain	N	Liver
Euthyroid	12	9.7 ± 0.9	13	5900 ± 700
Hyperthyroid	13	7.3 ± 0.8	13	3000 ± 400†
Euthyroid	5	19.1 ± 4.7	5	4900 ± 200
Hypothyroid	5	15.3 ± 2.7	5	1500 ± 200‡
Thyroid replacement	5	21.6 ± 2.4	5	4800 ± 400

* Activity is expressed in pmoles of 2-methoxyestradiol formed/mg of microsomal protein/10-min incubation.
† $P < 0.01$, different from control.
‡ $P < 0.001$, different from control.

severely hypothyroid rats, estrogen-2-hydroxylase activity declined in the liver (Table 4). Replacement doses of L-thyroxine in the thyroidectomized animals restored the hepatic enzyme levels to the normal range.

DISCUSSION

While estrogen-2-hydroxylase appears to be widely distributed among various tissues in the male rat, most of its activity is localized to the liver. In the mature female rat, enzyme activity was not detectable in the ovary or in the virginal uterus, although the placenta and fetus have measurable estrogen-2-hydroxylase activity. The wide range of enzyme activity seen in the various experiments in brain may be partially explained by the fact that in brain this enzyme increases with age (unpublished observations). Barbieri *et al.* [10], in an independent study, also found widespread distribution of the enzyme, and noted minimal activity in ovarian microsomes. In our previous paper [6], we reported higher levels of brain estrogen-2-hydroxylase than we now find. Our earlier calculations were in error because of a systematic underestimation of microsomal protein content, and our current values are in close agreement with those of Barbieri *et al.* [10].

Estrogen-2-hydroxylase activity has been reported previously in the rat brain [7, 15, 16], human placenta [17], human neoplastic breast tissue [18], and human fetal brain and pituitary [19]. Estrogen receptors have been shown to be present in numerous tissues, including brain [20], pituitary [20], liver [21], heart [22], lung [23], placenta [23] and kidney [24]. Since these tissues are able to convert estrogen to the catechol derivative *in vitro*, it is possible that 2-hydroxyestrogens are involved in modulating the effect of estrogen in these various target organs. Moreover, microsomal enzymes can bind catecholestrogens and convert them into reactive, possibly toxic, intermediates [25].

Unlike some other microsomal P-450-dependent enzymes, estrogen-2-hydroxylase activity in brain and liver could not be induced by 3-MC, PB, or testosterone. The activities of the brain and liver enzymes, however, are dependent upon testosterone, since castrated males showed dramatic falls in enzyme activity. Barbieri *et al.* [10] also showed a loss of hepatic enzyme activity with castration, and demonstrated that testosterone replacement restored enzyme activity to normal. No change in brain enzyme activity was noted with castration by these investigators. The female liver has significantly less activity than the male, a common dimorphism in hepatic steroid-metabolizing enzymes [26–28]. When the animals are matched for age, the males also have higher brain estrogen-2-hydroxylase activity.

Hypothyroidism and hyperthyroidism had identical effects, decreasing the enzyme activity in liver but not in brain. The divergent response of the brain and liver enzymes is not unexpected, since their different apparent K_m values indicate that they have different properties. The finding that both a deficit and a surfeit of L-thyroxine decrease rat hepatic enzyme activity has been reported for other androgen-dependent microsomal enzymes [29]. Benzo[*a*]pyrene hydroxylase activity, for example, is halved in hypothyroid rats and decreased by one-third when supraphysiologic doses of L-thyroxine are given [30]. While the explanation for this phenomenon is unclear, the decreased hepatic enzyme activity may represent a nonspecific response to illness *per se*. In thyroxine-treated rats total cytochrome P-450 may have increased activity, however, and other routes of metabolism may be stimulated at the expense of 2-hydroxylation.

In a small series of women with thyroid disease, urinary 2-hydroxyestrogen levels were found to be low in hypothyroidism and increased in hyperthyroidism [3]. The discrepancy with our results in hyperthyroidism may be due to sex or species differences, or to possible alterations in renal clearance in thyroid disease. An association between hypothyroidism and breast cancer has been postulated for many years [31]. Since many breast neoplasms are estrogen sensitive and in light of the observation that catecholestrogens act as anti-estrogens [1, 32] in certain systems, a decrease in total estrogen-2-hydroxylation seen in thyroid disease may provide a clue to the link between thyroid disease and breast cancer.

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