J. steroid Biochem. Vol. 22, No. 1, pp. 135–139, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

STUDIES ON OESTROGEN-2/4-HYDROXYLASE ACTIVITY IN MAMMALIAN BRAIN, USING A RADIOENZYMATIC ASSAY METHOD

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(*Received* 7 *May* 1984)

Summary—The activity of oestrogen-2/4-hydroxylase in brain tissue was studied using a radioenzymatic assay which controlled for non-specific formation of catechol oestrogen. The ontogeny of enzyme activity in male and female rat brain was examined between 1 and 70 days of age. Until 10 days of age, both sexes showed low enzyme activity. Between days 10 and 15 enzyme activity in the female significantly increased but was unchanged in the male. Male brain enzyme activity increased between days 15 and 20 whereas activity was unchanged in the female. Both males and females showed increases in enzyme activity between 20 and 25 days of age. Thereafter enzyme activity plateaued but at all ages between day 20 and day 70 male brain enzyme activity was significantly greater than in the female.

A study of the distribution of the oestrogen-2/4-hydrolase in the sheep hypothalamus revealed that the stalk/median eminence had approx 10 times greater activity than either the anterior, medial or posterior hypothalamus. These findings are discussed in relation to sexual differentiation of the brain.

INTRODUCTION

Hydroxylation of oestrogens at carbons 2 and 4 has been a recognized pathway of oestrogen metabolism for 20 years [1] and high concentrations of the enzyme oestrogen-2/4-hydroxylase have been identified in the liver of many species [2].

More recently this enzyme has been found in brain tissue [3, 4, 5, 6] and this has the highest oestrogen-2/4-hydroxylase activity of any tissue other than the liver [7]. Characterization of the brain enzyme has indicated that it is a microsomal NADPH-dependent, cytochrome P_{450}-linked monooxygenase [4].

The A-ring hydroxylated oestrogens (catechol oestrogens) are rapidly cleared from the circulation by *O*-methylation by the widely distributed enzyme catechol-*O*-methyl transferase (COMT) and therefore they are unlikely to act as circulating hormones. This emphasizes the importance of the hydroxylase in the brain, where locally synthesized catechol oestrogens may mediate or modulate the effects of oestrogens.

One method which has been used to measure oestrogen-2/4-hydroxylase activity is a radioenzymatic assay (REA) first described by Paul *et al.*, (1977) [4]. In this assay, the labile catechol oestrogens synthesised *in vitro* are rapidly converted to stable radiolabelled 2- and 4-*O*-methylated derivatives by coupling the hydroxylation of oestrogen with COMT in the presence of ^{3}H-S-adenosyl methionine (^{3}H-SAM). This method does not distinguish between 2- and 4-hydroxylation and we therefore follow the convention [8] of describing the enzyme as oestrogen-2/4-hydroxylase. Previous reports on the 2/4 hydroxylase enzyme using the REA may have to be reassessed in the light of recent evidence [9] which indicates that 4-hydroxylation of oestrogen is a non-enzymatic process. This contribution could seriously affect the determination of the relatively low activity of the brain 2/4-hydroxylase and suitable controls should be incorporated in the REA to avoid measurement of the non-specific conversion. Since the addition of reduced pyridine nucleotide cofactor had no effect on oestrogen hydroxylation at the C4 site, Fishman and Norton[9] have suggested that a "no cofactor" assay blank is a suitable control. We have therefore re-examined the activity of oestrogen-2/4-hydroxylase under assay conditions where the non-enzymatic transformation of oestrogen has been minimized.

Having established a modified REA, we have studied the ontogeny of the oestrogen-2/4-hydroxylase enzyme in rat brain. In addition, we have examined the localization of the enzyme within the hypothalamus of the sheep. It is hoped that these studies will provide new insights into the physiological significance of catechol oestrogen formation in the mammalian brain.

EXPERIMENTAL

Materials

NADPH, oestrogen (E_2), polyoxyethylene sorbitan mono-oleate (Tween 80), borate buffer solution and catechol-*O*-methyl transferase were purchased from Sigma Chemical Company, Poole, Dorset; ascorbic acid, magnesium chloride and *n*-heptane from B.G.H. Ltd, Dagenham, Essex; sucrose from Fisons Ltd, Leics.; [^{3}H]S-adenosyl methionine (7.8 Ci. mmole^{-1}) from New England Nuclear, Southampton; scintillator 299^{Tm} scintillation fluid from United Technologies, Packard, U.S.A.: authentic

1,3,5(10)-estratriene-2,3,17β-triol (2-OHE_2) and 2-hydroxy-estradiol 2-methyl ether (2-$MeOE_2$) from Steraloids Ltd, Croydon, England. Catchol-*O*-methyl transferase was also prepared in the laboratory using the following method:

Preparation of COMT

Rat livers (100 g) were homogenized in 4 vol of ice cold 0.9% KCl using an UltraTurrax (Janke and Kunkel Ltd) homogenizer. The homogenate was centrifuged at 78,000 *g* at 4°C for 30 min in an MSE susperspeed 50 centrifuge. All further procedures were also conducted at 4°C. The supernatant fraction was titrated to pH 5 with 1 M acetic acid and, after 20 min, the precipitate was centrifuged off at 14,000 *g* for 10 min. Protein fractionation of the supernatant was carried out using ammonium sulphate (BDH Aristar grade). The 0–30% precipitate was discarded and the 30–55% precipitate was re-dissolved in 50 ml of 1 mM sodium phosphate buffer (pH 7). The enzyme solution was dialysed for 12 h against 10^4 volumes of 1 mM sodium phosphate buffer containing 0.1 mM dithiothreitol. The final product was centrifuged to remove precipitates and, after dividing into 1 ml aliquot volumes, was stored at −20°C.

Preparation of tissues

Male and female Wistar rats aged between 1–80 days were housed under controlled lighting conditions (Lights on 05:00h–19:00h) and allowed free access to food and water. The sexually mature females were sacrificed on the day of oestrus. The animals were killed by decapitation between 10:00h and 11:00h and the brains (minus cerebellum) were dissected out and immediately placed into ice cold 0.32 M sucrose. Tissues were homogenized in 4 vol of 0.32 M sucrose for 15 s using an UltraTurrax homogeniser. Microsomal fractions were prepared by centrifugation at 11,000 *g* for 20 min and recentrifugation of the resultant supernatant at 105,000 *g* for 1 h. The microsomal pellet was redissolved in 0.1 M Tris–HCl buffer (pH 7.4) containing 0.1% Tween 80. Enzyme activity was 2–3 times greater in the presence of Tween 80 confirming the observation of Hersey and Weisz [8]. Sheep hypothalamus were obtained fresh from the abattoir and microsomes were prepared as above. All assays were performed on freshly prepared microsomes.

Enzyme assay

Reactions were carried out in 15 ml glass stoppered tubes. Microsomal protein (30–500 μg) in 50 μl buffer was incubated in a medium containing 50 μl of 0.1 M Tris–HCl buffer (pH 7); 0.1% Tween 80; 10 μl of 1 M $MgCl_2$, 2 μl of [^{3}H]S-adenosyl methonine, 20 μl of partially purified COMT or 1–10 i.u. of Sigma COMT; 5 μl of 50 mM L-ascorbic acid; oestradiol-17 (10–600 μM) in 2 μl ethanol and 5 μl of 12 mM NAPH. Blanks consisted of incubation medium and microsomal protein but without NADPH.

Standard curves were prepared by dissolving authentic catechol oestrogens in methanol and incubating in the assay medium in the absence of microsomes and NADPH. Reactions were started by placing the tubes in a water bath at 37°C. After 10 min reactions were stopped by the addition of 0.5 ml of 0.5 M borate buffer (pH 10.0). The radiolabelled product was extracted by Vortex mixing with 6 ml *n*-heptane for 1 min, followed by separation of the organic phase by centrifugation at 1000 *g* for 3 min. Two ml of the organic phase were transferred to scintillation vials and evaporated to dryness at 70°C under a stream of air. Twelve ml of scintillation fluid were dispensed into each vial and radioactivity counted in a scintillation counter. Each sample was assayed in duplicate with two "no cofactor" blanks. Microsomal protein was measured by the method of Lowry *et al.*,[10]. The enzyme activity was expressed as pmoles of product formed/mg protein/10 min.

Identification of the radiolabelled product was achieved by thin layer chromatography. The organic phase containing the reaction products was evaporated at 70°C under a stream of air, and the residue redissolved in 20–50 μl of methanol. Ten μg of authentic 2-methoxy oestradiol (2$MeOE_2$) was added as a carrier. Thin layer chromatography was performed on pre-coated silica gel plates (60F254, E. Merck, Darmstadt, Germany). The solvent systems used were (1) chloroform–methanol–acetic acid (96:3:1, by vol) and (2) dichloromethane–acetone (80:20, v/v). Following development, plates were scraped at 1 cm intervals into 15 ml stoppered glass tubes containing 3 ml ethanol. Tubes were Vortex mixed for 30 s and 0.5 ml of ethanolic supernatant was transferred to scintillation vials containing 12 ml scintillation fluid and monitored for radioactivity. Spots were visualized by spraying plates with Brady's solution (50% H_2SO_4–ethanol 1:1 saturated with 2,4-dinitrophenol).

Results were compared initially by an *F*-ratio test and then by Duncan's multiple range test [11] or by Student's *t*-test.

RESULTS

Identity of radioactive product

Using the two TLC solvent systems the radioactive product extracted after incubation of brain microsomes, co-migrated with standard 2 $MeOE_2$ (system 1 $R_f = 0.52$, system 2 $R_f = 0.43$).

Activity of COMT

When commercially available COMT (Sigma Chemical Co.) was employed in the REA, no 2 $MeOE_2$ could be isolated at the end of the assay. However COMT partially purified from rat liver was active, and, when the assay was performed in the absence of microsomal protein, did not show endogenous 2/4-hydroxylase activity.

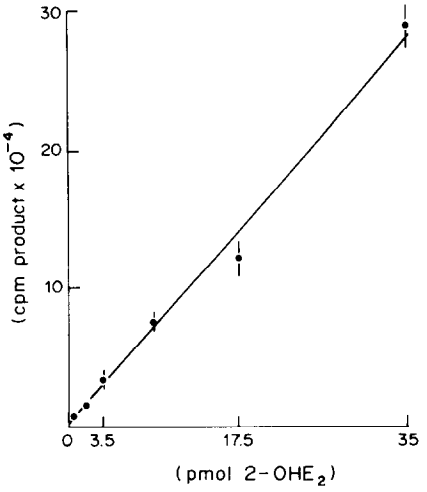

Fig. 1. Assay of catechol oestrogen using standard 2-OHE$_2$. Each point is the mean ± SEM of 6 assays. The line was fitted by regression analysis ($y = 27963x + 172$ $r = 0.97$).

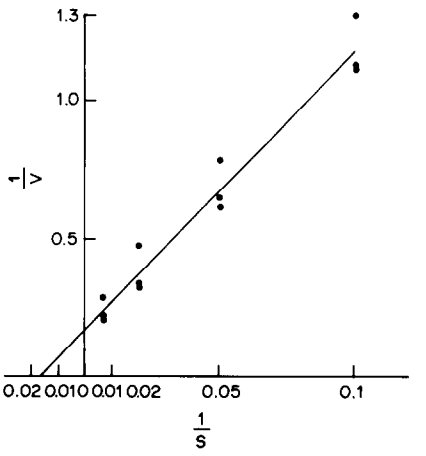

Fig. 3. Lineweaver–Burke plot of the substrate/velocity curve for the oestrogen-2/4-hydroxylase enzyme using E_2 concentrations up to 100 μM. v = reaction velocity (pmol·mg^{-1}·10 min^{-1}). S = E_2 concentration (μM). The line was fitted by regression analysis ($y = 10.1x + 0.17$, $r = 0.98$). The calculated K_m value is 60 μM.

Assay of standard 2-hydroxyoestradiol

Figure 1 shows the mean of 6 REAs using standard 2-OHE$_2$. The assay is linear up to 350 pmol 2-OHE$_2$ (data not shown). Assay sensitivity, defined as twice the blank (no 2-OHE$_2$) value is 0.05 ng·10 min^{-1} or 0.2 pmol·10 min^{-1}.

Kinetics of rat brain oestrogen-2/4-hydroxylase

The kinetics of rat brain enzyme were studied and a typical substrate/velocity curve is shown in Fig. 2. The total apparent reaction velocity increased with increasing concentration of E_2 to a plateau at about 300 μM. The non-specific blank (no cofactor) increased linearly however and when this was subtracted from the total to give the enzyme reaction velocity this was found to reach a plateau at 150 μM after which activity was inhibited by higher E_2 concentrations. Subsequent experiments were therefore performed using a substrate concentration of 150 μM E_2. Lineweaver–Burke analysis (Fig. 3) indicated that the apparent K_m for E_2 of the brain enzyme is 60 μM.

Ontogeny of oestrogen-2/4-hydroxylase in rat brain

The ontogeny of oestrogen-2/4-hydroxylase activity in the brain of male and female rats is shown in Fig. 4 with a statistical analysis of the results in Table 1. Until 10 days of age, male and female brain enzyme activity was low and showed no sex difference. In female animals activity increased significantly ($P < 0.05$) between days 10 and 15, however the increase observed in males did not reach statistical significance. Between days 15 and 20 male brain enzyme activity significantly increased ($P < 0.05$) but female enzyme activity was unchanged. Activity in both male and female brain significantly increased ($P < 0.05$) between days 20 and 25. Thereafter, en-

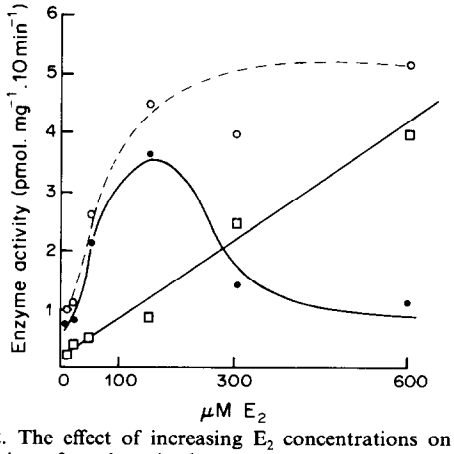

Fig. 2. The effect of increasing E_2 concentrations on the formation of product, in the presence (○) or absence (□) of NADPH. Subtraction of blank (no NADPH) values from the values with NADPH added, gives the calculated enzymatic activity (●). Each point represents the mean of triplicate determinations. SEM did not exceed the area covered by the symbols.

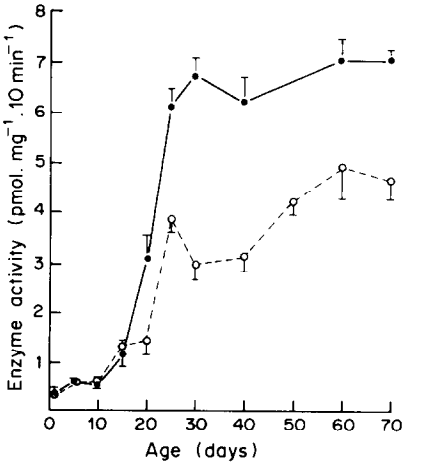

Fig. 4. Oestrogen-2/4-hydroxylase activity (pmol·mg^{-1}·10 min^{-1}) measured in brain (minus cerebellum) of male (●) and female (○) rats during development. Results are mean ± SEM of 6 (♂) and 5 (♀) determinations of each age. A statistical analysis of these results is given in Table 1.

Table 1. Ontogeny of oestrogen-2/4-hydroxylase activity in rat brain: statistical analysis

Age (days)	Oestrogen-2/4-hydroxylase activity (pmol·mg^{-1}·10 min^{-1}) Male	Female
1	0.38 ± 0.07^{a}	0.37 ± 0.02^{a}
5	0.57 ± 0.09^{ab}	0.63 ± 0.05^{a}
10	0.51 ± 0.05^{ab}	0.61 ± 0.03^{a}
15	1.16 ± 0.16^{b}	1.30 ± 1.18^{b}
20	$3.10 \pm 0.41^{c*}$	1.44 ± 0.27^{b}
25	$6.10 \pm 0.34^{d*}$	3.90 ± 0.26^{d}
30	$6.71 \pm 0.35^{d*}$	3.00 ± 0.29^{c}
40	$6.20 \pm 0.46^{d*}$	3.10 ± 0.24^{c}
50	—	4.24 ± 0.25^{de}
60	$7.00 \pm 0.40^{d*}$	4.88 ± 0.64^{e}
70	$7.00 \pm 0.18^{d*}$	4.65 ± 0.36^{e}

All values are mean ± SEM of $n = 6$(♂) and $n = 5$ (♀) determinations for each age.
Within-sex differences:
In any column, values with the same superscript are not significantly different $P > 0.05$ (Duncan's Multiple Range test).
Between-sex differences:
*Enzyme activity in male brain was significantly higher than that in the female ($P < 0.01$) from day 20 to day 70 inclusive (Student's t-test).

zyme activity plateaued but at all ages between day 20 and day 70 the activity of the male brain enzyme was significantly greater than the female, ($P < 0.05$ Student's t-test).

Table 2 shows oestrogen-2/4-hydroxylase activity in areas of sheep hypothalamus. The anterior, medial and posterior hypothalamus had relatively low enzyme activity which were not significantly different from each other. The stalk/median eminence region had a significantly higher activity ($P < 0.001$) this being approx 10 times that of the rest of the hypothalamus.

DISCUSSION

Studying the kinetics of the brain oestrogen-2/4-hydroxylase enzyme revealed that the non-enzymatic hydroxylation of oestrogen increased with increasing substrate concentration, whereas the enzymatic transformation showed saturation before decreasing at substrate concentrations above 150 μM. These findings are in accord with Fishman and Norton[9] who, using a radiometric assay, also observed a decrease in the velocity of the enzymatic reaction at high substrate concentrations. In addition, using rabbit hypothalamic microsomes, Hersey *et al.*,[12] obtained a decrease in reaction velocity at oestrogen concentrations greater than 100 μM. The exact mechanism of this decrease is unknown but it may be due to product inhibition. Because of it, a substrate concentration of 150 μM was used for the enzymatic hydroxylation of oestrogen in our REA.

The apparent K_m for the adult male brain enzyme (60 μM) is similar to but slightly lower than previous reports (98 μM, Hoffman *et al.*[7] and 140 μM, Hersey *et al.*[12]).

Using this modified REA was have examined the ontogeny of the brain oestrogen-2/4-hydroxylase activity in male and female rats. In both sexes enzyme activity increased with age. The first significant increase in brain oestrogen-2/4-hydroxylase activity occurred in female rats between 10 and 15 days of age. The physiological significance of this increase is unknown but it may be relevant to our understanding of the mechanisms of sexual differentiation of the brain. Androgenization of female rats can be achieved before the tenth day of life [13]. Oestrogens (which can be synthesised in the brain by aromatization of androgens [21]) are more potent in this respect than the parent C_{19}-androstene compounds, whereas A-ring reduced metabolites are ineffective [14, 15]. This has led to the hypothesis that the effects of androgens on sexual differentiation of the brain are mediated by oestrogen formed by aromatization within the hypothalamus [16] (in the female, target areas in the brain are protected from the action of oestrogens by specific circulating binding proteins [17]). The observed increase in 2/4-hydroxylation of oestradiol in the female brain after day 10 of life, may explain the attenuation in the response to androgen administered at this time. On the other hand, 2-hydroxy oestradiol induces defeminization when given to neonatal female rats [18] but high pharmacological doses were used in this study.

Brain oestrogen-2/4-hydroxylase activity continued to increase in both sexes levelling off after day 25. Male enzyme activity was significantly greater than in the female from day 20 of life onwards. Sexual dimorphism in brain oestrogen-2/4-hydroxylase activity has also been found by Hoffman *et al.*,[7] but Barbieri *et al.*,[6] were unable to show this. The reasons for these divergent results are unclear as these authors used the same age and species of rat and similar assay methods. We find a lower oestrogen-2/4-hydroxylase activity in adult male rats (5–7 pmol mg^{-1} 10 min^{-1}) compared to the two earlier reports (15 pmol mg^{-1} 10 min^{-1}) which

Table 2. Distribution of oestrogen-2/4-hydroxylase activity in the sheep hypothalamus

Region	Oestrogen-2/4-hydroxylase activity (pmol·mg^{-1}·10 min^{-1})
Anterior hypothalamus	0.98 ± 0.39 ($n = 5$)
Medial hypothalamus	0.81 ± 0.48 ($n = 5$)
Posterior hypothalamus	0.52 ± 0.45 ($n = 5$)
Stalk/median eminence	10.93 ± 3.90 ($n = 4$)*

Results are mean ± SEM and were analysed using Student's t-test.
*Oestrogen-2/4-hydroxylase activity in SME microsomes was significantly greater than in other hypothalamic areas ($P < 0.001$).

did not employ suitable controls for the non-enzymatic formation of catechol oestrogens and are thus probably overestimates.

There have been few reports on the distribution of oestrogen-2/4-hydroxylase activity within the brain. Fishman and Norton[3] found activity in hypothalamus of ovariectomized female rats but not in cortex whereas Ball *et al.*[5] showed only slight differences in the 2- and 4-hydroxylating capacities of hypothalamus, cortex, pituitary gland and hippocampus. One measurement of 2/4-hydroxylase activity in male rat median eminence revealed that this tissue had a greater enzyme activity than the hypothalamus [19]. In the human foetus one study found an even distribution of 2/4-hydroxylase activity in the brain [20] but in another [19] greater activity was found in pituitary gland and hypothalamus when compared to cortex and limbic tissue. Little weight can be assigned to these studies however as, in general, the numbers of observations are low and the variation in activities are high.

In our examination of the hypothalamic distribution of oestrogen-2/4-hydroxylase in the sheep, the SME had approx 10 times the activity of the rest of the hypothalamus. This finding is particularly interesting as the medial basal hypothalamus has also been identified as a site of relatively high aromatase activity in the brain [21]. Furthermore, subcellular fractionation has shown that both aromatase and oestrogen hydroxylase are microsomal enzymes [22]. It is possible therefore that the actions of oestrogens formed locally by aromatization of androgens could be modulated by the extent of local 2- and 4-hydroxylated oestrogen formation. Whether, in this context, the latter represents an activating or inactivating process is unknown at present.

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