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2-HYDROXYLATION OF ESTROGENS IN THE BRAIN PARTICIPATES IN THE INITIATION OF THE PREOVULATORY LH SURGE IN THE RAT

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SUMMARY

Estradiol-2-hydroxylase, the enzyme responsible for the conversion of estrogens to catechol estrogens was measured in the brain of female rats at specific stages of the estrus cycle. Radiometric measurements of the enzyme activity in microsomal, mitochondrial, and synaptosomal fractions of the brain revealed a sharp increased in activity at proestrus just prior to the preovulatory LH surge. The enzyme activity declined to lower levels at diestrus and metestrus. No comparable fluctuations were noted in the liver enzyme. These changes in brain enzyme activity in conjunction with demonstrated positive feedback of exogenous catechol estrogens on pituitary LH release, suggest that a rise in endogenous catechol estrogen formation in the brain may be responsible for the physiological induction of the preovulatory LH surge.

The neuroendocrine mechanisms underlying the biphasic regulation of pituitary gonadotropin secretion by estrogens are still obscure. The observation that central nervous system tissues are capable of converting estrogens to 2-hydroxyestrogens (1-4) stimulated speculation that this transformation was involved in the physiological estradiol-gonadotropin regulatory process. This concept received support from subsequent demonstrations that 2-hydroxyestrogens are effective inhibitors of enzymes involved in both the biosynthesis (5) and the metabolism (6) of biogenic catecholamines in the brain providing for a biochemical link between the estrogens and the neurotransmitters most directly involved in the hypothalamic control of gonadotropin release (7). Administration of pharmacological doses of 2-hydroxyestrone was reported to result in augmented plasma LH concentrations in both male (8) and female rats (9) suggesting that the catechol estrogens could be mediating the positive feedback action of estradiol. These latter experiments, however, are faced with the uncertainty as to whether exogenously administered or even

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endogenous 2-hydroxyestrogens of peripheral origin necessarily mimic the physiological function of the same substances generated in situ in central tissues. To examine the role of brain 2-hydroxyestrogens in the neuroendocrine mechanisms underlying the physiologic control of gonadotropin secretion in the female rat we therefore sought to measure the activity of the brain enzyme responsible for the 2-hydroxylation at the various stages of the estrus cycle. The purpose of the study was to detect any variations in the enzyme activity and their relationship to the other hormonal changes of the estrus cycle.

Measurement of the estradiol-2-hydroxylase enzyme activity is difficult because of the exceptional lability of its catechol estrogen products. Two procedures have, however, been devised which mitigate or avoid this problem and permit assays of the enzyme activity in various tissues. One is a radioenzymatic procedure which depends on the conversion of the 2-hydroxylated estrogen products to the more stable labelled-O-methylated derivatives by means of the catechol-O-methyltransferase enzyme and the use of S-adenosylmethionine containing a labelled methyl group as the methyl donor (10). The other procedure is a radiometric method in which 2-^{3}H-estradiol is utilized as the substrate and the tritiated water generated is used as the index of enzyme activity (1). In this radiometric assay which has recently been validated the hydroxylation reaction proceeds without an isotope effect or a NIH shift (12) so that stoichiometry prevails. In our studies we elected to use the latter procedure in part because of its greater sensitivity which allows for the use of substrate concentrations closer to physiological levels.

In the initial studies we sought to measure enzyme activities in subcellar fractions of the whole brain with the exception of the cerebellum. Previously, using the radioenzymatic procedure other investigators reported (3, 10) that the brain estradiol-2-hydroxylase is a particulate enzyme distributed in the microsomal and mitochondrial fractions, with the bulk being present in the microsomes. In the experiments reported here the activity of the enzyme in

the microsomal, mitochondrial, and synaptosomal fractions of the rat brain was evaluated at each stage of the 4 day ovulatory cycle.

MATERIALS AND METHODS

Female CD rats obtained from Charles River Laboratory with regular 4 day estrus cycles were housed under conditions of 7:30 a.m. - 7:30 p.m. light and 7:30 p.m. - 7:30 a.m. dark. Estrus cycles were monitored by vaginal cytology. Groups of six animals were sacrificed by cervical dislocation at 3 p.m. of metestrus, 10 a.m. of diestrus, 2 p.m. of proestrus and 10 a.m. of estrus. Trunk blood samples were collected for hormone determinations and the brains and liver portions were dissected. The whole brain with the exception of the cerebellum was rapidly homogenized in 0.32 M sucrose and the subcellular fractions were separated by standard procedures. The liver microsomal and mitochondrial preparations were obtained similarly.

Incubations of each subcellular fraction were carried out with 0.25-0.75 nmoles of 2-^{3}H-estradiol (20 Ci/mM) in 0.1 M Tris buffer pH 7.2 at 37° in the presence of NADH and NADPH for 60 minutes. The incubations were frozen and then lyophilized and the specific activity of the water lyophilizate was determined. The protein content of the incubated tissues was measured by the Lowry procedure.

The blood samples from each animal were analyzed for their LH, estradiol, and progesterone concentration to validate the cycle stage determinations. The LH (13), progesterone (14), and estradiol (15) measurements were carried out by conventional radioimmunoassays using defined antisera. The hormone assay results are listed in Table 1, and indicate that the proestrus samples were obtained when preovulatory estradiol levels were at or near maximum but prior to the initiation of the LH surge.

RESULTS AND DISCUSSION

The results of the estradiol-2-hydroxylase activity measurements at the various stages of the cycle in each of the three brain subcellular particulate fractions are recorded in Figure 1. In each of the subcellular fractions there is a very large multifold rise in enzymic activity at proestrus from that found in the preceding diestrus stage. The changes are most dramatic in the microsoma fraction where the diestrus to proestrus rise is sixfold. With the exception of the diestrus samples, microsomal enzymic activity was uniformly higher than the mitochondrial or synpatosomal fractions. At all stages the synaptosomal enzyme content exceeded that of the mitochondrial although some contamination of the former by the latter cannot be excluded. In all particulate fractions the enzyme activity continued to be elevated at estrus, diminished greatly at metestrus and decreased further to a nadir at diestrus. It needs to be stressed that the greater endogenous content of estradiol in the proestrus

TABLE 1

SERUM HORMONE CONCENTRATIONS AT TIME OF BRAIN ENZYME ASSAY

	LH (ng/ml)	PROGESTERONE (ng.ml)	ESTRADIOL (pg/ml)
Diestrus	26 ± 14*	28 ± 9	28 ± 12
Proestrus	47 ± 11	9 ± 3	102 ± 37
Estrus	29 ± 8	9 ± 2	24 ± 7
Metestrus	75 ± 43	23 ± 10	22 ± 5

*Mean ± S.D. (n = 6)

brain relative to the tissues from the other stages of the cycle would serve to dilute the radiolabelled substrate and at substrate saturating conditions result in an underestimation of enzyme activity by the radiometric procedure. Thus, although an accurate correction for this effect is not possible at this

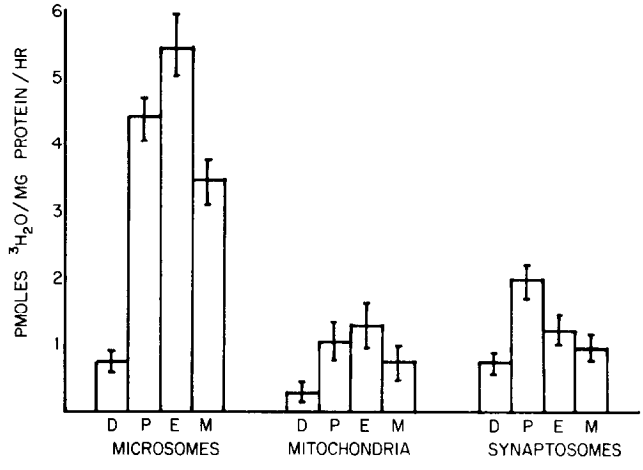

FIGURE 1: Estradiol 2-Hydroxylase Activity in Rat Brain Subcellular Fractions in Different Stages of the Estrus Cycle. D = diestrus, P = proestrus, E = estrus, M = metestrus. Incubations were carried out in 0.1 M Tris HCl buffer pH 7.2 under air at 37°C for 60 minutes. Each incubation contained tissue (1 mg protein.c.c.), NAD (1 µM/c.c.), NADP (1 µM/c.c.), glucose-6-phosphate dehydrogenase (1 u/c.c.), glucose-6-phosphate (15 µM/c.c.) 2-^{3}H-estradiol, 20 Ci/mM (0.05 nM/c.c.). Incubation volumes were 5-15 c.c. depending on quantity of tissue available. At termination incubations were flash frozen and lyophilized1 1 c.c. samples of lyophilized water were counted in triplicate in a Packard scintillation counter. Control incubations were carried out as above except that the tissue was omitted; values obtained were treated as blanks and substracted from tissue incubation results. Results are presented as means of separate determinations (n = 4) with the bars representing standard deviations. The proestrus and estrus values were significantly different from diestrus in all subcellular fractions ($p < .01$). Metestrus values were significantly greater than diestrus in the microsomal and mitochondrial fractions ($p < .01$, $p < .05$ respectively).

time, it is likely that such a correction would increase the observed proestrus enzyme activity to levels exceeding those at estrus. It is possible that, because the endogenous estrogen content in the synaptosomal fraction is minimal, thereby eliminating this dilution effect, this fraction exhibits higher activity at proestrus relative to estrus. It is also possible that the timing of the experiment failed to coincide with the maximum of enzyme activity at proestrus which could be considerably higher than that observed at estrus. In sharp contrast to the large changes in estradiol-2-hydroxylase in the female rat brain during the estrus cycle the liver microsomal and mitochondrial enzyme activities remained at a constant level throughout (Figure 2) emphasizing the uniqueness of the changes in brain enzyme levels.

The enzymatic changes reported here, taken in conjunction with the previous evidence of stimulating feedback effects of exogenous catechol estrogens on LH release (8,9) provide experimental support for the concept that the great increase in brain 2-hydroxyestrogen formation at proestrus participates in the initiation of the ovulatory LH surge. The impact of the 2-hydroxyestrogens

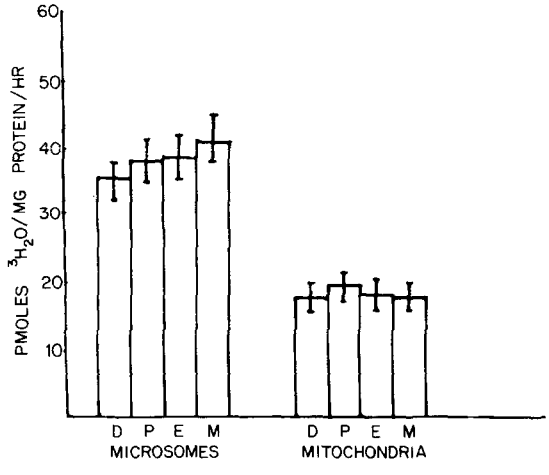

FIGURE 2: Estradiol-2-Hydroxylase Activity in Rat Liver Subcellular Fractions in Different Stages of the Estrous Cycle. The incubations were performed precisely as with the brain preparations. The results are the means of 4 determinations. The results obtained in the various stages of the cycle were not significantly different.

on LH secretion is apparently not exerted at the pituitary level, since 2-hydroxyestrone has been reported not to sensitize pituitary cell cultures to the effects of LHRH (16). It is more likely that the increased estrogen 2-hydroxylation in hypothalamic sites at proestrus acts to induce the LHRH release which in turn triggers the pituitary LH surge (17). The present results constitute the first report of a major fluctuation in estradiol metabolism in the rat brain during the estrus cycle. Their temporal pattern suggests that a direct relationship exists between the increased 2-hydroxylation of the female hormone in the brain and several neuronal events occurring on proestrus. These include not only the preovulatory discharge of LHRH (17), but also the induction of lordosis behavior (18), the stimulation of running activity (19), and the inhibition of food ingestion (20). It is, of course, possible that the increased 2-hydroxylation and these events are not interrelated and that the rise in enzymatic activity reflects the modulation of a metabolic disposition pathway to accommodate the increasing hormone concentrations at proestrus. The observation, however, that no such induction occurs in the liver weighs against such a rationalization. Estradiol has been reported to be a better substrate for the brain 2-hydroxylase than estrone (10), although by radiometric measurements both estrogens are equally good substrates. Whether the specific 2-hydroxyestrogen responsible for the LH release is 2-hydroxyestrone or 2-hydroxyestradiol or whether these are transient precursors of the actual active substance is presently unclear. Equally unknown is whether this interaction is accomplished through catecholaminergic mechanisms or through receptor mediated events (21) or by other as yet unknown modalities. The elucidation of these questions remains a fertile field for investigation.

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