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INDUCTION OF UTERINE PEROXIDASE: CORRELATION WITH ESTROGENIC ACTIVITY

P. H. JELLINCK and ANNE-MARIE NEWCOMBE
Department of Biochemistry, Queen's University, Kingston, Ontario K7L 3N6, Canada

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SUMMARY

A good correlation was obtained between the uterotrophic activity of a number of steroids and related compounds and their ability to induce peroxidase in the immature rat uterus *in vivo*. It is proposed that this biochemical test gives a better indication of an intact receptor–acceptor system in target cells than assays based on the binding to high affinity estrogen receptors.

INTRODUCTION

It has been shown previously [1] that a peroxidase that catalyzes the metabolism and binding of estradiol to protein and other high-molecular weight substances can be induced in the uteri of immature rats by physiological doses of estradiol or by pregnant-mare-serum gonadotrophin. There is substantial evidence [2–6] that the enzyme is produced *in situ* and is distinct from the peroxidase brought to the uterus by eosinophils [7]. The uterine enzyme has been partially purified [8] and its subcellular location has been determined [9]. The relationship between the quantity of nuclear estrogen–receptor complex formed and the amount of peroxidase induced by estradiol has also been investigated, together with the effect of the antiestrogen, Nafoxidine [10].

The present paper shows the correlation between the estrogenic activity of various steroids and their ability to induce peroxidase in the rat uterus after *in vivo* administration.

EXPERIMENTAL

Materials. Crystallized and freeze-dried bovine serum albumin, estrone, estradiol-17β and 17α, testosterone propionate, estriol(1,3,5(10)-estratriene-3,16α,17β-triol), mestranol(17α-ethynyl-3-methoxy-1,3,5(10)-estratriene-17β-ol) and hexestrol(*meso*-3:4-*di*(*p*-hydroxyphenyl)hexane) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A, and 2,4-dichlorophenol and guaiacol from Eastman Kodak Co., Rochester, NY, U.S.A. 2-Hydroxyestradiol(1,3,5(10)-estratriene-2,3,16α,17β-tetrol) was generously supplied by the Cancer Chemotherapy National Service Center, Bethesda, MD, U.S.A. and dihydroxyhexestrol(*meso* - 3:4 - *di*(*p* - hydroxyphenyl) - 1:16-dihydroxyhexane) [11] by Mr. W. Lawson (Courtauld Institute of Biochemistry, London, U.K.). Dr. T. Nambara (Tohoku University, Senda, Japan) kindly provided 3-deoxyestrone(1,3,5(10)-estratrien-17-one) and 2-hydroxy-3-deoxyestradiol (1,3,5(10)-estratriene-2,17β-diol). [4-^{14}C]-estradiol (40 mCi/mmol) from Schwarz/Mann, Orangeburg, NY, U.S.A. was shown by chromatography and autoradiography to be free of radioactive impurities. It was diluted with carrier to a specific radioactivity of 2–3 mCi/mmol and kept at 4°C in the dark as a stock solution in ethanol (1 mg/ml). The diethyl ether used for extraction was free of peroxides.

Preparation of tissue extract. Immature female Holtzman rats (24–28 days old) weighing 70–90 g were injected subcutaneously with various doses of the test compounds dissolved in sesame oil 18 h before killing the animals. The uteri were dissected free of adhering fat, blotted and weighed. The tissue was then cut into small pieces and homogenized in 5 ml of 0.1 M sodium phosphate buffer, pH 7.0 in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was diluted to 11 ml with phosphate buffer, centrifuged at 100,000 *g* (4°C) for 30 min and, after washing with buffer, the sediment was rehomogenized in the appropriate amount of 1.2 M NaCl to give a 5% (w/v) homogenate. It was centrifuged again at 100,000 *g* for 30 min and portions of the supernatant fraction (1.2 M NaCl extract) used for the subsequent enzyme assays.

Determination of peroxidase activity. The uterine extract (0.5 ml) derived from 25 mg wet wt. of tissue was incubated with constant shaking for 30 min or 1 h at 38°C with [4-^{14}C]-estradiol (1.8 μM), 2,4-dichlorophenol (0.25 mM), H_2O_2 (0.25 mM) and bovine serum albumin (10 mg) in 0.1 M sodium phosphate buffer, pH 7.4; total vol. 4 ml. After incubation, the medium was extracted three times with equal vol. of peroxide-free ether and the radioactivity in the aqueous fraction was determined by scintillation counting as described previously [12]. Peroxidase activity was also determined by the more direct method using guaiacol as substrate [13]. The reaction mixture (3 ml) contained guaiacol (13 mM) and H_2O_2 (0.33 mM) in 0.01 M sodium phosphate buffer (pH 7.0) and 0.05–0.2 ml of the fraction containing peroxidase depending on its activity. The linear increase in absor-

Table 1. Effect of treatment with estrogens and related compounds on the induction of uterine peroxidase in immature rats

Compound*	Dose (μg)	% Of added ^{14}C in aqueous medium after extraction with ether: Incubation time 30 min	Incubation time 1 h	Rate of oxidation of guaiacol ΔE_{470}/min/g tissue
Control (oil)	—	6.3	7.8	0
Hexestrol	5	46.9	50.9	19.2
Estradiol-17β	5	25.6	36.5	8.0
Mestranol	5	20.2	25.8	4.6
Estrone	5	18.8	27.5	5.6
	10	50.6	56.6	18.4
Estriol	10	15.1	25.5	3.6
	25	26.7	42.6	9.2
2-Hydroxyestradiol	10	9.4	15.3	1.9
	25	13.9	30.5	6.8
	50	27.1	38.2	10.0
	100	34.3	41.7	11.0
Estradiol-17α	25	10.6	12.6	0.8
	50	14.0	17.4	0.8
Dihydroxyhexestrol	10	4.6	6.4	0.1
	100	11.1	19.3	0.3
3-Deoxyestrone	100	7.4	8.6	0
2-Hydroxy-3-deoxyestradiol	100	6.2	7.5	0
Testosterone propionate	100	7.2	9.2	0
2,4-Dichlorophenol	1000	5.9	8.4	0

* Arranged in order of uterotrophic potency [24–26]. Uterine extracts from 25 mg of tissue were incubated with [4-^{14}C]-estradiol or with guaiacol as described in the text. The compounds tested were given by subcutaneous injection in oil 18 h before the assay. Results are the mean of two experiments using pooled uteri from 3–5 rats.

bance at 470 nm resulting from the oxidation of guaiacol was then followed at 25°C in a Unicam SP 800 recording spectrophotometer.

RESULTS AND DISCUSSION

The effect of a number of estrogens, both natural and synthetic, on the induction of uterine peroxidase in immature rats is shown in Table 1. A good correlation was obtained between the estrogenic (uterotrophic) potency of the compounds tested and their ability to induce peroxidase measured by two different biochemical methods. Thus an increase in the metabolism of [4-^{14}C]-estradiol and also in the rate of oxidation of guaiacol by uterine preparations was produced by small doses of estradiol-17β or hexestrol administered *in vivo*. Mestranol, estrone, estriol and 2-hydroxyestradiol required higher doses to show this effect, while testosterone propionate, 3-deoxyestrone, 2-hydroxy-3-deoxyestradiol, dihydroxyhexestrol and 2,4-dichlorophenol which are devoid of estrogenic activity, did not induce the enzyme. It has also been shown that progesterone does not induce uterine peroxidase and that the response to estradiol is influenced by the endocrine state of the rats [14]. Peroxidase activity determined by the guaiacol assay was proportional to the percentage conversion of [^{14}C]-estradiol to water-soluble products after 30 min of incubation (Fig. 1). All attempts to induce uterine peroxidase *in vitro* have so far been unsuccessful.

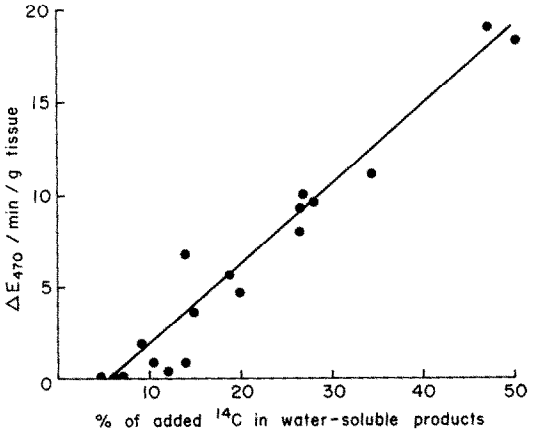

Fig. 1. Correlation curve between methods of assay for uterine peroxidase. Enzymic activities were determined either by measuring the percentage conversion of [^{14}C]-estradiol to water-soluble products after 30 min or by the oxidation of guaiacol, as described in the text. The ^{14}C-measurements in the aqueous fraction are not corrected for control values (~ 6%).

It is generally accepted that a prerequisite for the expression of uterotrophic activity by any estrogen is its binding to the estrogen receptor in the cytosol of the target tissue cells [15]. A large number of estrogens and their analogues have now been examined for both their uterotrophic activity and binding by the uterine receptor and a considerable degree of correspondence between these two parameters was observed [16]. The specificity of the estrogen receptor

in the human uterus has also been studied with a number of steroids [17]. Highest affinity to the receptor was found if the steroid had a phenolic hydroxyl group on C-3 and an alcoholic hydroxyl group on C-17 in the β-configuration. The presence of the free phenolic hydroxyl on ring A was essential and its position of critical importance. Our results with 3-deoxyestrone, estradiol-17α and 2-hydroxy-3-deoxyestradiol support these findings. The discrepancy with mestranol which shows low receptor binding affinity [16] can be explained by the *in vivo* demethylation of this 3-methoxy steroid to ethynylestradiol [18] which would not occur in the *in vitro* systems in the absence of the liver [19]. Conversely, 2-hydroxyestradiol which is one of the major products of estradiol biotransformation in man [20] showed relatively low peroxidase inducing activity in agreement with its low uterotrophic potency and yet possesses high affinity for the estradiol receptor [21]. Thus our assay of estrogenic activity based on the induction of peroxidase gives a more appropriate measure of the physiological characteristics of the steroids than those based on binding affinity to specific receptors. It is also easy to perform and, with the limited number of compounds tested, gives a quantitative index of estrogenic activity.

Binding to the estradiol cytosol receptor is merely one step in the complex mechanism of gene activation by estrogens [15]. The biosynthesis of an end-product like peroxidase or the "induced protein" [22, 23] as a result of treatment with hormone gives a better indication of an intact receptor–acceptor system in the target cells. It also provides an alternative assay to the various biological techniques already in use to determine estrogenic activity.

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