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Urinary excretion pattern of catecholestrogens in pre-ovulatory LH surge during the 4-day estrous cycle of rats

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ABSTRACT. The formation of catecholestrogens represents a major pathway of estrogen metabolism and catecholestrogens are regarded as the main estrogen metabolite in non-pregnant state of various mammalian systems. In the present investigation, level of 2-hydroxyestrone, the major catecholestrogen excreted in rat urine, was measured by radioimmunoassay following acid hydrolysis and column chromatography of the 24-h urine samples of female Sprague Dawley non-pregnant rats during their 4-day estrous cycle. Urinary levels of estrone, estradiol and estriol were measured. Unlike the plasma level, urinary 2-hydroxyestrone showed a marked increase during the pre-ovulatory LH surge suggesting a plausible role of catecholestrogen in the mid-cycle elevation of the gonadotropin level in normal cycling rats.

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INTRODUCTION

Studies on estrogen metabolism have shown that 2-hydroxylation leading to the formation of catecholestrogens is the major pathway in both animals and humans (1-4). These catecholestrogens exhibit marked anti-estrogen activity (2, 5), anticarcinogenic effect (4, 6), antioxidant property (7, 8) and their protective actions on atherosclerosis and osteoporosis may be ascribed to their inhibition of leukotriene synthesis (9). The primary catecholestrogen, 2-hydroxyestrone (2-OHE_1) has a reduced uterotrophic activity and significant effect on gonadotropin and PRL secretion (10-12). It is well established that 2-OHE_1 in pharmacological doses stimulates the secretion of LH and FSH (13, 14). Suppression of PRL secretion by 2-OHE_1 in both rats and humans is a well-known fact (15, 16). Two-hydroxyestrogens have been shown to interact with estrogen receptor of the anterior pituitary and hypothalamus and they may modify the action of catecholamines by competition for dopamine receptors or inhibition of catechol-O-methyltransferase (COMT) and/or tyrosine hydroxylase (3, 17). The feedback effects of estrogen on these processes has been further suggested to be mediated by 2-hydroxyestrogens (2).

The role of catecholestrogens in human parturition and rat pregnancy has been investigated earlier in this laboratory and 2-OHE_1 has been reported to play an important part (18-20). Catecholestrogens represent the main estrogen metabolites in the non-pregnant state (21). Chattoraj *et al.* (22) and Ball *et al.* (21) have reported mid-cycle surges in urinary 2-OHE_1 levels in women. During previous investigation in our laboratory on plasma 2-OHE_1 and gonadotrophin levels determined by radioimmunoassay during the estrous cycle of rats, no significant change in plasma 2-OHE_1 concentration has been observed during the pre-ovulatory LH surge (23). Therefore, it was felt pertinent to undertake the present study which reports the urinary levels of the catecholestrogen 2-OHE_1, estrone (E_1), estradiol-17β (E_2) and estriol (E_3) determined by radioimmunoassay (RIA) using antisera raised in our laboratory and chromatographic methods, throughout the 4-day estrous cycle of rats. Correlation of these estrogens with the pre-ovulatory LH surge has been investigated with special reference to a possible role of 2-OHE_1 in mid-cycle elevation of the gonadotrophin level.

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MATERIALS AND METHODS

Materials

Radioactive and inert 2-OHE_1 were synthesized and stored according to the method described by Chattoraj *et al.* (22). All radioactive materials were purchased from New England Nuclear, Boston, MA, USA. Organic solvents used were all glass distilled. Sephadex LH-20 used for column chromatography was obtained from Pharmacia Fine Chemicals, Piscataway, NJ, USA. The phosphate-saline buffer (0.1 M Na-phosphate, 0.9% NaCl, 0.1% Na-azide, 0.1% g-globulin fraction II, pH 7.2) used in the procedure contained 0.05% ascorbic acid for the protection of catecholestrogens (22). Dextran coated charcoal was prepared by adding 0.5 g dextran T-70 (Pharmacia Fine Chemicals) to 5 g Norit A (Fisher Scientific Co., Pittsburgh, PA, USA) in 40 ml buffer and was diluted to 10 mg charcoal/0.2 ml prior to use. Other steroids and catecholamines were purchased from Steraloids Inc., Wilton, NH, USA and Sigma Chemical Co., St. Louis, MO, USA. Radioactivity was counted in 10 ml Liquiscint scintillation cocktail obtained from National Diagnostics, Somerville, NJ, USA.

Preparation of antisera

Each 6-week-old New Zealand white male rabbit received 1.0 mg of the 2-OHE_1-17-(O-carboxymethyl) - oxime coupled to bovine serum albumin conjugate, emulsified in 0.5 ml of complete Freunds adjuvant (Calbiochem, La Jolla, CA, USA). The animals were injected intra-dermally once a week for four weeks and then boosted every four weeks. The anti-2-OHE_1 rabbit antisera used throughout the study was obtained following the eleventh booster. A final dilution of 1:1500 giving 40-50% binding was used for the study. The specificity of the antibody was determined by the degree of cross-reactivity with other steroids and catecholestrogens according to the method of Chattoraj *et al.* (22). The cross-reactivity test was done by RIA. Scatchard plot data (not shown) was obtained by the addition of 0.005-1.0 ng of 2-OHE_1 to tubes containing antisera and 3000 cpm of radiolabeled 2-OHE_1. Bound and free steroids were separated by dextran-coated charcoal as described for the assay of 2-OHE_1. The percent cross-reactivity for various steroids and catecholamines reported in Table 1 was the measure of percent inhibition for a standard arbitrary dose of competitor.

Animals

Adult female Sprague Dawley rats weighing about 175 g obtained from Charles River Breeding Labs, MA, USA were used for this study. The rats were kept in metabolic cages under controlled conditions of temperature, humidity and light (lights on 06:00 h - 18:00 h). Estrous cycles were monitored by checking vaginal smears daily in the morning.

Table 1 - *Cross-reactivity data of different steroids and catecholamines against anti-2-hydroxyestrone antisera raised in rabbits.*

Test compounds	Cross-reactivity (%)[a]
2-hydroxyestrone	100
2-hydroxyestradiol	100
2-hydroxyestriol	20
Estrone	0
Estradiol-17β	0
Estriol	0
Epinephrine	0
Dopamine	0
Pregnanediol	0
Cortisone	0

[a]Cross-reactivity test was done by the method according to Chattoraj *et al.* (22).

Collection of urine and blood

Rats were grouped according to their stages in the estrous cycle and 24 h urine samples were obtained throughout the cycle. All urine samples were collected in sterile containers with 10% ascorbic acid in 2N HCl from 09:00 h to 09:00 h next day. Rat urine is slightly alkaline. Therefore, ascorbic acid was added to protect the catecholestrogens from being oxidized. Aliquots were kept frozen at -20 C until analyzed.

For collection of blood, rats were grouped according to the hours and stages of the estrous cycle and 4-6 rats were sacrificed from each group at 3 h intervals from 09:00 h to 24:00 h. The blood samples were collected into ice-cold tubes containing heparin and 20% aqueous ascorbic acid solution and immediately centrifuged at 4000 rpm at 4 C for 10 min. The plasma was preserved in 20% ascorbic acid, snap frozen in dry ice-acetone and stored at -20 C until analyzed.

Quantitation of 2-OHE_1

RIA was performed following acid hydrolysis and column chromatography of the urine samples according to the method of Chattoraj *et al.* (22) with some modifications. Urine samples were pooled according to the stages of the estrous cycle and duplicate I ml aliquots were hydrolyzed in a boiling water bath after addition of 0.1 ml 1.5 M potassium iodide, 0.1 ml sodium metabisulphite and 0.2 ml of

concentrated HCl. The acid hydrolyzed urine samples were cooled and neutralized with 10N sodium hydroxide and phosphate buffer (pH 7.2) containing 0.05% ascorbic acid. Approximately 1000 cpm of tritiated 2-OHE_1 was added for recovery, mixed, kept for 10 min and then extracted 3 times with anhydrous diethyl ether. The combined organic extracts were first washed with sodium bicarbonate buffer (pH 10.5) containing ascorbic acid, then with 10% acetic acid, evaporated under nitrogen at room temperature and dissolved in elution solvent. Column chromatography was performed using Sephadex LH-20 column (0.8x10 cm) and benzene: methanol (9:1) containing 0.005% ascorbic acid as elution solvent. The organic extract after evaporation to dryness under nitrogen and being dissolved in the elution solvent was applied to the column. Appropriate fractions were collected in disposable culture tubes. The overall recovery varied from 70 to 80%.

For RIA, duplicate standard solutions of 2-OHE_1 ranging from 0-1000 pg/ml in buffer were prepared. Additionally, 100 ng tubes were included in the assay for determination of non-specific binding. Approximately 8000 cpm of radioactive 2-OHE_1 in 0.1 ml buffer and 0.1 ml of diluted antisera were added to both sample and standard tubes. Following overnight incubation at 4 C, dextran-coated charcoal (10 mg/0.2 ml buffer) was added and the tubes were vortexed, left for 10 min at 4 C, centrifuged and decanted into scintillation vials for counting. Water blanks were included in each assay.

Plasma 2-OHE_1 was quantitated by radioimmunoassay following Sephadex LH-20 column chromatography in the same way as described above.

Estimation of 2-hydroxyestradiol

Plasma and urinary levels of 2-hydroxyestradiol (2-OHE_2) were quantitated according to the method of Chattoraj *et al.* (22). RIA was performed following acid hydrolysis of urine samples and isolation of the catecholestrogen by column chromatography of both plasma and urine samples, in the same way as described in the quantitation of plasma and urinary 2-OHE_1.

Estimations of plasma LH and urinary steroids

Plasma LH levels were determined in duplicate using the kits and standard procedure supplied by NIADDK (USA) as described in our previous report (23). The results were expressed as ng/ml of the NIADDK rat standard LH (0.03xNIH-LH-S1). Samples of a pool of plasma from hypophysectomized female rats were included in the assay to check the reliability of each assay. The intra and inter-assay coefficients of variation were found to be 5.6% (no.=8) and 7.9% (no.=5), respectively.

Urinary E_1, E_2 and E_3 were measured using our own antisera raised against their 17β - 6-CMO-BSA conjugates in rabbits. Following addition of radioactive estrogens for recovery, duplicate samples were extracted and chromatographed on Sephadex LH-20 column according to the method of Butcher *et al.* (24). After chromatography, RIA was performed as described before. The intra and inter-assay coefficients of variation were found to be 9.2% (no.=8) and 10% (no.=5) for E_1, 7.9% (no.=6) and 8.8% (no.=6) for E_2 and 6.7% (no.=5) and 10.2% (no.=5) for E_3, respectively.

RESULTS

Specificity of antisera raised against 2-OHE_1 investigated by determining the cross-reactivity of various steroids and catecholamines has been represented in Table 1. Apart from 2-OHE_1, only 2-OHE_1 (100%) and 2-hydroxyestriol (20%) have shown significant cross-reactivity. The specificity has been further ensured by a purification step before RIA. Addition of known concentrations of 2-OHE_1 to the acid hydrolyzed urine has rendered a linear regression with a correlation coefficient r=0.99. The intra-assay coefficient of variation has been found to be 8.7% (no.=7) and the inter-assay coefficient of variation, 10% (no.=6). Same procedure has been followed in the determination of 2-OHE_1 level in rat plasma, the intra-assay and inter-assay coefficients of variation being 8.2% (no.=5) and 6.1% (no.=5). The possibility of oxidation of 2-OHE_1 during the collection of urine and blood and subsequent processing has been minimized by adding ascorbic acid (22).

The temporal pattern of plasma 2-OHE_1 levels at 3-h intervals along with the plasma LH profile is shown in Figure 1. The concentration of 2-OHE_1 ranged from undetectable to 10 pg/ml, after subtracting the procedural blank. Plasma LH concentration remained at a low level during metestrus, diestrus and the morning of proestrus. Pre-ovulatory LH surge began at 12:00 h proestrus, reached a maximum at 15:00 - 18:00 h and again declined at 24:00 h.

Urinary 2-OHE_1, E_1, E_2 and E_3 profiles have been represented in Table 2. The concentrations of the estrogens E_1, E_2 and E_3 did not show any significant change among the different days of the cycle. However, urinary 2-OHE_1 levels in urine samples collected from 09:00 h. of proestrus, estrus, metestrus and diestrus showed significant elevation from the morning of proestrus through the morning of estrus ($p<0.005$, ANOVA; df=12) compared to the other three days of the cycle. Concentration of 2-OHE_2 was also determined by RIA since 100% cross-reactivity

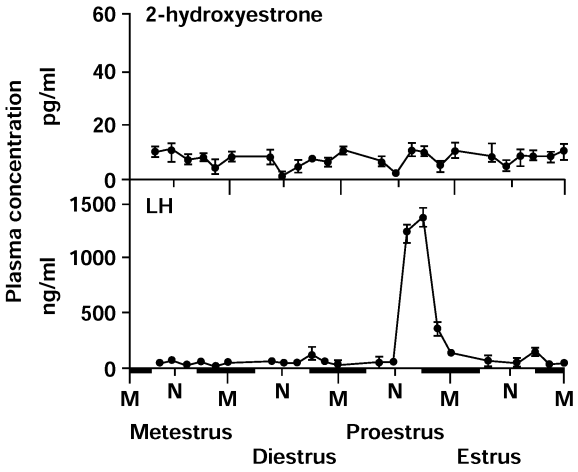

Fig. 1 - *Plasma concentrations (±SE) of 2-hydroxyestrone and LH, during the 4-day estrous cycle of rat. Measurements were taken at 3-h intervals. Each point represents a duplicate determination on pooled plasma from 4-6 rats. N: Noon; M: Midnight;* ——*: dark.*

Table 2 - *Urinary excretion of steroids throughout the 4-day estrous cycle of rate.*

Stages of estrous cycle	2-OHE$_1$[a]	E$_1$[b]	E$_2$[c]	E$_3$[d]
Metestrus	42.1±3.0	23.5±2.2	16.1±9.2	3.5±2.1
Diestrus	50.1±4.2	28.2±4.9	24 ± 5.1	3.9±7.8
Proestrus	67.2±5.4[f]	19.7±7.8	29.8±6.9	5.4±9.7
Estrus	34.9±9.2	21.9±5.0	25.9±3.1	6.1±3.9

[a]2-hydroxyestrone; [b]estrone; [c]estradiol-17β; [d]estriol; [e]all the values in the table expressed as nanograms per 24 hours±SE; [f]$p<0.005$ (ANOVA) compared to values in the metestrus, diestrus and estrus of the cycle.

was also shown by this important catecholestrogen with anti-2-OHE$_1$ antisera. We could not observe any detectable amount of 2-OHE$_2$ both in plasma and urine samples in our study.

DISCUSSION

In the present study, we have quantitated urinary as well as plasma 2-OHE$_1$ and tried to arrive at a possible correlation between the circulating 2-OHE$_1$ level and the plasma LH pattern throughout the 4-day estrous cycle of rat.

In any method for the measurement of catecholestrogens in body fluids, an effective means for protection of the oxygen sensitive catechol group during each step of the procedure must be the primary consideration. It has been clearly demonstrated that oxidative decomposition of 2-OHE$_1$ occurred not only during sample preparation but even during the period of incubation for RIA in an unprotected medium (22). In our study this problem was eliminated by using the buffer containing 0.05% ascorbic acid which fully protects 2-OHE$_1$ without interfering with the antibody-steroid interaction.

Specificity of the antisera raised against 2-OHE$_1$ was determined by cross-reactivity test of various steroids and catecholamines (Table 1). Apart from 2-OHE$_1$, the major cross reactant was 2-OHE$_2$ (100%), although 2-hydroxyestriol also showed some cross-reactivity. Considering the importance of 2-hydroxylation pathway in estrogen metabolism (1-4), we have tried to determine the level of 2-OHE$_2$, another important catecholestrogen, owing not only to its 100% cross-reactivity shown with anti-2-OHE$_1$ antisera but also to its capacity to interact with dopamine receptors in rat anterior pituitary (25) and suppress circulating prolactin levels in rats (26). A further purification step by Sephadex LH-20 column chromatography was included in order to isolate 2-OHE$_2$ from 2-OHE$_1$. No detectable amount of 2-OHE$_2$ was found both in plasma and urine samples indicating that, quantitatively, 2-OHE$_1$ appears to be by far the major 2-hydroxylated estrogen metabolite present in the samples.

The trends of plasma 2-OHE$_1$ and LH levels throughout the estrous cycle of rats (Fig. 1) corresponded well with the previous findings (23, 24, 27). Our data are in agreement with that reported by Fishman and Martucci (28). Although a high plasma 2-OHE$_1$ level was described by Paul *et al.* (29), the overestimation was suspected to be from the contamination of semipurified COMT preparation used in the radioenzymatic assay by estrogen 2-hydroxylase (30). Plasma 2-OHE$_1$ levels at 3-h intervals ranging from 0-10 pg/ml failed to show any significant variation at any time during the entire estrous cycle. Although a pre-ovulatory peak of small magnitude was shown by plasma 2-OHE$_1$ during early proestrus, it is very difficult to ascribe a physiological significance to this observation since similar elevations were also observed during late proestrus and estrus.

A well-defined trend for plasma 2-OHE$_1$ or a satisfactory correlation between concentration of 2-OHE$_1$ and mid-cycle LH elevation, did not emerge from our experiment, as regards the plasma catecholestrogen profile. Unlike all the hormones measured in previous investigations (23, 24), 2-OHE$_1$ did not show a consistent rise in concentration before or during the pre-ovulatory LH surge. The occurrence of low plasma 2-OHE$_1$ level might be due to the fact that plasma hormone levels are representatives at the sampling times employed and might not provide the necessary information as to the active estrogen metabolites like the 2-hydroxylated compounds, since the

latter, specially 2-OHE_1, exhibits a high metabolic clearance rate, about 100 times greater than that of E_2 (31). Any other reason could not be ascribed to this observation at this point. Apparently, blood levels cannot be used as a pointer for the elucidation of the physiological role of 2-OHE_1.

On the other hand a clear consistent rise has been observed in urinary 2-OHE_1 concentration from the morning of proestrus through the morning of estrus in our study. Earlier investigations in this laboratory reported the importance of 2-OHE_1 in human and rat pregnancy (18-20). It also represents the main estrogen metabolite in non-pregnant states (21, 22). Significance of urinary excretion pattern of this important catecholestrogen has already been established in pregnant and non-pregnant mammals (20, 22). Ball *et al.* (21) and Chattoraj *et al.* (22) reported the mid-cycle surges in urinary 2-OHE_1 level in women. Therefore, it was felt pertinent to quantitate 2-OHE_1 in rat urine. Subsequently, elevated level of urinary 2-OHE_1 during pre-ovulatory LH surge in the 4-day estrous cycle of rat, observed in our experiment, could very well corroborate the previous findings of Chattoraj *et al.* (22) and Ball *et al.* (21). The low plasma level and high urinary level of 2-OHE_1 during proestrus when considered together along with the high plasma LH concentration in the same stage of estrous cycle might confirm the quantitative importance of the 2-hydroxylation pathway in estrogen metabolism.

It has been observed from the present investigation that increase in estrogen production during proestrus is mainly reflected in the urinary level of 2-OHE_1 in the 4-day estrous cycle of rat. This might indicate a plausible role of this important catecholestrogen in participation in mid-cycle LH surge. Thus, the measurement of urinary level of 2-OHE_1 could serve as a good index of ovarian function in non-pregnant state in mammals and consequently the method developed in our experiment could provide a rapid, reliable and precise way of quantitation of the 2-hydroxylated products of estrogen metabolism.

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