Published in final edited form as:

Neuroendocrinology. 1989 August ; 50(2): 204–210.

Chronic Inhibition of Hypothalamic-Pituitary-Ovarian Axis and Body Weight Gain by Brain-Directed Delivery of Estradiol-17 β in Female Rats

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Abstract

The effect of preferential delivery of estradiol (E₂) into the brain on both the hypothalamic-pituitary-ovarian axis and weight gain was studied in female rats. When E₂ was coupled to a lipoidal dihydropyridine-pyridinium carrier, the resulting carrier E₂ complex (CE), upon a single intravenous administration to cycling female rats, caused a dose-dependent inhibition of ovulation which lasted 3 times longer than with uncoupled E₂. The dose of CE that delayed ovulation for 4 days was one twentieth the amount of E₂ needed to produce the same effect. Studies in ovariectomized (OVEX) rats indicated that the prolonged ovulation-blocking action of CE appeared to be related to a sustained storage and release of E₂ in the brain, which in turn suppressed the release of hypothalamic luteinizing hormone-releasing hormone (LHRH) and pituitary luteinizing hormone (LH). Upon single intravenous administration in pubertal female rats, CE caused a dose-dependent reduction of body weight gain for a minimum period of 28 days. The inhibitory action of CE on body weight gain was more potent and longer lasting than that of E₂ in pubertal rats. When administered in OVEX rats, CE produced a loss of body weight that lasted significantly longer than that produced by uncoupled E₂ in these rats. These results suggest that the biological action of E₂ can be potentiated by this novel chemical delivery system.

Keywords

Dihydropyridine carrier-bound estradiol; Estradiol; Ovulation; Luteinizing hormone; Luteinizing hormone-releasing hormone; Body weight

Constant exposure to E₂ inhibits the secretion of hypothalamic luteinizing hormone-releasing hormone (LHRH) and consequently reduces the release of pituitary luteinizing hormone (LH) and blocks ovulation [11, 12, 29]. Administration of E₂ with or without progestin has been applied as a contraceptive agent in females; however, the steroid has to be administered daily to be effective. Downregulation of the pituitary-ovarian axis by exogenous LHRH has been used to treat reproductive disorders including endometriosis and

premenstrual syndrome [20, 22]. The major limitations of this treatment are mental depression and altered thermoregulation due to E₂ deprivation in the brain [8] as well as accelerated bone loss due to peripheral E₂ deficiency [22].

In addition to the effects on reproduction, ovarian hormones modulate body weight and food intake behaviors in a variety of mammals including laboratory rats [33, 35]. Treatment with E₂ results in a transient decrease in food intake, with a long-lasting decrease in body weight and lipoprotein lipase activity; further, progesterone attenuates these E₂-induced effects in adult female rats [13, 14, 25, 26, 35, 36]. In pubertal female rats, E₂ also reduces the body weight gain but not food intake or locomotor behaviors [14, 34, 35]. The central nervous system is the proposed site of E₂ action [5, 34]; however, several reports indicate that E₂ also decreases body weight by direct peripheral alteration of lipid and/or carbohydrate metabolism [13, 24].

Recently a chemical delivery system for E₂ has been formulated based on interconversion of a lipophilic dihydropyridine and hydrophilic pyridinium salt carrier that is covalently linked to a steroid [4, 6]. Upon intravenous injection in rats, carrier E₂ complex (CE) has been shown to distribute throughout the body, including the brain, and oxidize rapidly to the corresponding pyridinium salt form in vivo [4, 6, 21]. The resulting quaternary E₂ salt (QES) has been shown to be rapidly excreted from the periphery while retained for a long time in the brain by the blood-brain barrier system [21]. QES is believed to slowly hydrolyze and release free E₂ within the brain. Thus, we investigated the potential usefulness of a long-acting brain delivery of E₂ as a model of 'hypothalamic castration'. We also evaluated the biopotency of CE, other than in reproduction, by comparing the action of CE and E₂ on body weight.

Materials and Methods

Animals

Pubertal female rats (30 days old), cyclic female rats (90 days old) and ovariectomized (OVEX) female rats (90 days old) of CD Sprague-Dawley strain were purchased from Charles River Laboratory (Wilmington, Mass.) and maintained under controlled lighting (lights on 05.00-19.00 h) and temperature (25 °C). The animals were provided with food pellets and tap water ad libitum. Experiments were performed on these rats after a minimum 5-day inhabitation in the controlled laboratory conditions or our animal quarters. The effect of steroids on reproduction was studied in cyclic female rats and OVEX female rats. The cyclic female rats exhibited at least two consecutive 4-day estrous cycles before experimentation. The effect of steroid on body weight gain was studied in OVEX rats (which were also used for the study on reproduction) and in pubertal female rats. Pubertal rats (105-114 g BW) were subjected to a treatment regime and their body weights recorded at 4-day intervals for 28 days. After appropriate treatment, OVEX rats (240-318 g BW; 2-3 weeks after OVEX) were sacrificed by decapitation at various time intervals. Animal weights were recorded prior to drug administration and before sacrifice.

Drug Administration

Both CE and dihydropyridine carrier (PR-230) were obtained from Pharmalec (Alachua, Fla.), and E₂ was obtained from Sigma (St. Louis, Mo.). These drugs were dissolved in dimethyl sulphoxide (DMSO; Sigma) prior to use. Between 09.00 and 10.00 h, animals were treated with a single drug injection into the jugular vein under ether anesthesia.

Tissue and Plasma Sample Collection

The action of CE or E₂ on estrous cycle length was studied by injecting the animals intravenously with various dosages of CE, E₂ or DMSO vehicle on the day of estrus. In the study in which the effect on ovulation was determined, animals were either injected intravenously with 7.7 µmol/kg of CE, 11.1 µmol/kg of E₂ or 1 ml/kg of DMSO on the day of estrus. These animals were surgically inspected for ova 2, 3 and 4 days after the drug treatment under ether anesthesia. The action of CE on pituitary LH secretion was determined by assaying LH levels in plasma samples collected for 10 days through a chronically implanted intra-alrial cannula [28] from the steroid-treated females. The effect of the steroids on hypothalamic LHRH, pituitary portal plasma LHRH and systemic plasma LH was studied in rats which were OVEX 2-3 weeks previously and received a single intravenous injection of 7.7 µmol/kg of CE, 7.7 µmol/kg of E₂ or 1 ml/kg of DMSO vehicle. Following 1 or 16 days after the drug administration, animals were anesthetized with Saffan (4–5 ml/kg i.p.; Glaxo) between 09.00 and 10.00 h. and peripheral blood samples were withdrawn for LH measurements. Pituitary stalks of these animals were then exposed [30] and portal blood samples collected Tor 60-90 min for LHRH radioimmunoassay (RIA). Hypothalamic tissues were obtained [27] after the blood collections and assayed for LFIRH. In a separate experiment, OVEX animals received a single-dose intravenous injection of CE (7.7 µmol/kg) E₂ (7.7 µmol/kg) or DMSO (1 ml/kg) between 09.00 and 10.00 h and brain tissues and trunk blood samples from these animals were obtained at 10 and 30 min. 1, 2 and 6 h, 1, 2, 4, 8, 12, 16, 20 and 24 days. Plasma from trunk blood samples were obtained and assayed for LH and E₂. Brain tissue samples were collected [27] and homogenized in 1 ml 0.01 M phosphate buffer saline (pH 7.5) and assayed for protein and E₂.

Radioimmunoassay

E₂ was extracted from tissue and plasma samples by adding recovery counts (900 cpm of ³H-E₂) and using hexane:ethyl acetate (3:1) extraction and Celite column separation (extraction recoveries were from 70 to 90%). The column fractions were dried, resuspended and assayed [1] using a specific E₂ antiserum which was raised against E₂ conjugated at C6 (No. 614; CE cross-reactivity was < 1.0%. The assay sensitivity was 1.6 pg/tube, and the intra- and interassay coefficient of variation were 11.8 and 14.7%, respectively. LH and LHRH levels were measured by double antibody RIA [30] using the NIADDK LH RIA kit and LHRH antiserum (CRR11B73), respectively. The intraassay coefficient of variation for LHRH was 9%, for LH it was 6%. The concentration of tissue protein was determined using BCA protein assay reagents (Pierce Chemical Co., Rockford, Ill.).

Statistics

The level of hormones, estrous cycle length and changes in body weight between the treatment groups were compared by analysis of variance and Duncan's multiple range test. Comparison between growth curves was made by using analysis of covariance and Fisher's protected least square difference procedure [17]. A p value < 0.05 was considered significant.

Results

Effect of Steroids on Reproduction

Administration of CE in cyclic female rats lengthened the estrous cycle in a dose-dependent fashion (fig. 1). Furthermore, CE was found to be approximately 3 times more effective than an equimolar dose of E_2 ; the dose of CE ($0.128 \mu\text{mol/kg}$), which effectively lengthened the estrous cycle from 4 to 8 days, was one twentieth the amount of E_2 ($2.56 \mu\text{mol/kg}$) needed to produce the same effect. Neither the vehicle (DMSO; 1 ml/kg) nor the carrier (PR-230: $7.7 \mu\text{mol/kg}$) affected the length of the estrous cycle (fig. 1). Steroid-treated rats (CE - $7.7 \mu\text{mol/kg}$, $n = 6$; E_2 - $11.1 \mu\text{mol/kg}$, $n = 7$) showed no ova in the oviducts on days 2, 3 and 4, but the control rats did show ova (12 ± 1 , $n = 6$) on day 4 (day of estrus) after DMSO treatment. Hence, the lengthening of the estrous cycle following CE or E_2 treatment was due to inhibition of ovulation. Whether or not the observed inhibition of ovulation in female rats was due to reduction of pituitary secretion of LH was determined by assaying LH levels in plasma samples, collected each afternoon (15.00-18.00 h) for 10 days, following an intravenous injection of CE ($7.7 \mu\text{mol/kg}$), E_2 ($11.1 \mu\text{mol/kg}$) or DMSO (1 ml/kg) alone on the day of estrus. None of the CE- or E_2 -treated rats showed any LH surge ($< 4 \text{ ng/ml}$) for a minimum period of 10 days, while control rats showed the LH surge ($21 \pm 6 \text{ ng/ml}$; $n = 6$) on the afternoon of proestrus 3 days after the treatment. The absence of the LH surge for 10 days, together with the lengthening of the estrous cycle in the steroid-treated animals (cycle length; CE = 25 days, E_2 = 12 days), suggested a sustained inhibition of LH release in these rats.

Treatment with CE ($7.7 \mu\text{mol/kg}$) in OVEX rats also produced a significant reduction ($p < 0.05$) of plasma LH levels (fig. 2c, 3e), and the duration of LH inhibiting action of CE was 6 times longer than that achieved by an equimolar dose of E_2 (fig. 3e). The reduction of LH secretion paralleled the reduction of LHRH secretion into pituitary portal blood of steroid-treated OVEX rats (fig. 2). Moreover, the inhibitory action of CE on LHRH release lasted longer than that of E_2 . Hypothalamic LHRH concentrations 1 and 16 days after CE treatment and 1 day after E_2 treatment were increased when portal plasma levels of LHRH were reduced. Measurement of E_2 levels in brain tissue and plasma samples following CE or E_2 ($7.7 \mu\text{mol/kg}$) treatment on OVEX rats revealed that E_2 clearance is much slower in CE-treated rats than in E_2 -treated rats (fig. 3a-d). The levels of E_2 in brain tissues following CE treatment stayed elevated 4-5 times longer than after E_2 treatment. When the relative concentrations of brain E_2 and plasma E_2 of CE-treated rats were compared (fig. 3a-d), steroid levels between 1 and 16 days after treatment were more than 12-fold higher in brain samples ($12\text{-}76 \text{ ng/g}$ protein or $1.2\text{-}7.6 \text{ ng/g}$ wet tissue, approximately 10% protein was commonly observed in brain tissues) than in plasma samples ($0.6\text{-}0.03 \text{ ng/ml}$). When the

kinetics of hormone concentration and biological action were compared, the levels of E₂ in the brain were inversely associated with the levels of LH in plasma (fig. 3a-c, e).

Effect of Steroids on Body Weight

In intact pubertal rats, treatment with 2.56-51.2 µmol/kg of CE produced a dose-dependent reduction ($p < 0.01$) in the rate of body weight gain when compared with DMSO vehicle (1 ml/ kg)-treated control (fig. 4). Pubertal rats which were treated with the carrier (PR-230; 12.8 µmol/kg) alone, however, showed a similar rate of weight gain as DMSO-treated rats. Since the growth curve of PR-230-treated rats overlapped with the growth curve of DMSO-treated rats, the data were not presented in figure 4. Treatment with 25.6 µmol/kg E₂ also reduced the rate of body weight gain for a period of 12 days ($p < 0.01$) in pubertal rats. After day 12 the Entreated rats showed an accelerated gain of body weight, and by day 20 body weights of these rats were no longer different from those of control rats. It is noteworthy that none of the CE-treated groups showed any growth 'recovery', and by the end of the observation period body weights of these rats were still significantly lower than those of DMSO- or E₂-treated rats. Comparison of the growth curve between CE- and E₂-treated rats revealed that CE is more potent than E₂ in reducing body growth in pubertal rats.

In OVEX rats, treatment with 7.7 µmol/kg of CE induced a reduction in body weight that lasted for a minimum period of 24 days (fig. 2). An equimolar (7.7 µmol/kg) dose of E₂ caused a transient loss in body weight for 4 days, followed by a reduction in body weight gain until day 12. After day 12, Entreated rats showed a weight 'recovery', and by day 20 the body weight of these rats was no longer different from the DMSO-treated controls.

Discussion

These results suggest that the carrier-bound E₂ preferentially delivered E₂ into the brain where it became 'locked' in, causing a sustained suppression of LHRH and LH release and ovulation longer than the uncoupled E₂ in female rats. Furthermore, the present results indicated that the brain-directed delivery of E₂ sustained the E₂-induced inhibition of body weight in intact and in OVEX female rats. The persistence of the body weight response to CE also appeared to be related mainly to a sustained storage and release of the steroid in the brain. Hence, the biological action of E₂ can be potentiated by this novel chemical delivery system.

The data shown in figure 1 indicated that CE is more potent and long-acting than a comparable amount of unbound E₂ in lengthening the estrous cycle days. Lengthening of the estrous cycle days in the steroid-treated rats appeared to be due to reduction of pituitary LH secretion and inhibition of ovulation as determined in cyclic female rats following treatment with a higher dose of the steroid. These results are consistent with the previously observed sustained inhibition of LH release by CE in gonadectomized male and female rats [3, 6] and CE-induced long-term stimulation of sexual behaviors in male rats [2]. In OVEX rats, the reduction in plasma LH secretion paralleled the reduction of LHRH secretion into pituitary portal blood following CE or E₂ treatment (fig. 2). Furthermore, the action of CE on LH and LHRH release lasted longer than after E₂ treatment. Hypothalamic LHRH concentrations 1 and 16 days after CE treatment and 1 day after E₂ treatment were increased when portal

plasma levels of LHRH were reduced. The increase in hypothalamic LHRH levels observed after steroid treatments can be attributed to the buildup of the tissue hormone concentrations resulting from reduced hormone secretion [28]. It has been shown previously that, although E₂ initially reduces the pituitary LH responsiveness to LHRH, the continued presence of elevated E₂ levels augments pituitary sensitivity in rats [16, 18]. These results suggest that the long-term inhibition of ovulation and LH release by CE was due primarily to sustained suppression of LHRH secretion from the hypothalamus.

As shown in figure 3, E₂ levels in brain tissue following CE treatment remained elevated 4-5 times longer than after E₂ treatment. Therefore, it appeared that a substantial amount of the CE was “locked” in the brain for a prolonged period of time. During the 1st day, free E₂ levels in brain tissues of CE-treated rats were severalfold lower than those in E₂-treated rats. The assay method used in this study detected E₂ but not CE. Hence, the majority of the steroid in brain tissues during, at least, the 1st day in CE-treated rats was not in E₂ form, but possibly was in CE form. Steroid levels between 1 and 16 days after CE treatment were more than 12-fold higher in brain samples than in plasma samples. Furthermore, previous studies have shown that the plasma clearance of both CE and QES were rapid, and tissue half-life of QES was 5- to 10-fold higher for brain tissues than for kidney, liver, lung, heart, testes, eye and fat tissues [4, 6, 21]. It appears, therefore, that the prolonged elevation of E₂ in plasma following CE treatment is primarily the result of a continuous leakage of E₂ from the brain into the periphery. The possibility that some of the peripheral E₂ in CE-treated rats may have come from a storage source other than the brain cannot be ruled out. When the kinetics of hormone concentration and biological action were compared, the levels of E₂ in the brain were inversely associated with the levels of LH in plasma (fig. 3a-c, e). Together these observations suggest that CE administration promoted sustained E₂ storage and release in the brain, resulting in increased biological action of the steroid. It has been shown previously that estrogens substituted at C17 position like CE do not bind to steroid receptors [10]. Therefore, it appears that the E₂ which is released chronically from the conjugate rather than CE and QES affects LHRH secretion. Further studies to determine the displacement of hypothalamic E₂ receptors by labeled CE and QES are required to resolve this issue.

Unlike E₂, the CE treatment maintained moderate levels of E₂ both in the brain and plasma during the contraceptive interval. Hence, CE treatment could effectively inhibit the hypothalamic-pituitary-ovarian axis while maintaining other E₂-dependent brain activities and bone turnover [8, 22]. We have shown that a small single dose of CE (0.125 µmol/kg) is sufficient to effectively block ovulation for one estrous cycle length. Also, recently Anderson et al. [3] have demonstrated inhibition of LH in OVEX rats following a similar CE dose regime. Furthermore, they have shown that the biopotency of CE is increased 3 times if the drug was administered repeatedly at 2-day intervals. Thus, it may be possible to produce the contraceptive action of CE by multiple administration of a very small dose of the drug. Studies in the monkey have demonstrated that repeated CH injections produced no significant neurotoxicity in the brain [7]. In light of these findings, the possible application of CE in combination with progestin as a long-acting contraceptive and as an agent for ‘hypothalamic castration’ in patients with certain reproductive diseases should be explored.

Furthermore, since E₂ affects a variety of other brain functions [19, 37], usage of CE beyond those of reproductive cyclicity and disorders should also be explored.

Sustained body weight reduction after administration of CE in female rats (fig. 4, 5) is consistent with similar findings in OVEX rats and also with the observed chronic suppression of the hypothalamic-pituitary-gonadal axis in female rats (fig. 1-3). The long-term suppression of body weight by CE was observed both in intact pubertal females and OVEX adult females. In weaning rats it has previously been shown that E₂ reduces body weight without affecting the food intake or running wheel activity [14, 34, 35]. Similarly, in OVEX rats, the changes in food intake have been shown to be of little importance to the persistent weight loss and altered adiposity following E₂ administration [14, 33]. It has been proposed that multiple or diverse effects of the sex hormone on various metabolic processes are more important than behaviors in determining body weight and composition [35]. E₂ is known to affect energy expenditure and macronutrient processing primarily through an action on the brain [23, 32]. The steroid can also affect food utilization efficiency by altering pituitary trophic hormone-mediated homeostatic processes [9, 15]. Results shown in figure 3 indicate that sustained elevated concentrations of free E₂ are present in the brain as long as 24 days after treatment with CE. Furthermore, brain half-life of QES following CE treatment has been shown to be higher than the peripheral half-life [4, 6, 21]. Thus, the potentiation of the weight-reducing action of E₂ following CE treatment in young intact females and adult OVEX rats appeared to be mainly related to the central action of the steroid. The possibility remained that part of the action of CE on body weight was also related to the peripheral action of E₂, since plasma levels of E₂ were moderately elevated as a result of brain leakage after the CE treatment in OVEX rats. Further studies with CE and antisera to E₂ are needed to explore relative contributions of peripheral and central regulatory mechanisms controlling body weight.

In conclusion, these results indicated that the biological effect of E₂ on reproduction and body weight can be potentiated by this novel chemical delivery system of the steroid, and that the increased biopotency of CE appeared to be mainly related to sustained storage and release of E₂ in the brain.

Acknowledgements

We thank Dr. Romi Roy for technical assistance; Pharmatec for providing CE and PR-230; Dr. A.F. Parlow, Pituitary Hormones and Antisera Center & NIADDK for LH RIA kits; Dr. V. Ramirez (or LHRH antiserum, and Glaxo for Saffan. This work was supported partly by a grant from Gynex and by NIH grants AG-05453 and HD-20498. Preliminary results of this investigation were presented at the 17th Annual Meeting of the Society for Neuroscience, November 1987. New Orleans, La.

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Fig. 1.

Dose-response relationship of the action of CE (●; 0.13-38.5 μmol/kg) and E₂ (■; 0.26-38.5 μmol/kg) on estrous cycle length in cyclic female rats. Control rats received carrier PR-230 (▨; 7.7 μmol/kg) or DMSO (□; 1 ml/kg) vehicle alone. Data are mean + SE of 4-10 rats. The x-axis is in log scale. Data of CE-treated and E₂-treated groups were significantly different ($p < 0.05$) from DMSO-treated and PR 230-treated groups. The values in CE-treated rats were significantly different ($p < 0.01$) from E₂-treated rats at the same dose treatment.

Fig. 2. Effect of CE (7.7 $\mu\text{mol/kg}$), E₂ (7.7 $\mu\text{mol/kg}$) or DMSO (1 ml/kg) alone on hypothalamic LHRH (a), pituitary portal plasma LHRH (b) and plasma LH (c) in OVEX rats. Values are mean + SE of 5-8 rats. * $p < 0.05$, compared to DMSO-treated groups.

Fig. 3.

Comparison of E₂ concentrations in brain tissues (a-c) and plasma (d), and of LH levels in plasma (e) of OVEX rats which were treated with CE (●; 7.7 µmol/kg). E₂ (□; 7.7 µmol/kg) or DMSO (△; 1 ml/kg) alone. The y-axis is in log scale. Data shown are mean ± SE of 5-7 rats. The levels of E₂ in brain tissues and in plasma of CE-treated rats were significantly different ($p < 0.05$) than those in E₂-treated rats at the same time point, with exception at 4 h, 6 h and 20 days for hypothalamic tissue, 6 h for preoptic tissue, 4 and 6 h for cortical tissue, and 4 h, 6 h and 20 days for plasma. E₂ values in DMSO-treated rats were similar at 1 h and 4 days. E₂ values in DMSO-treated rats, when combined, were significantly different ($p < 0.05$) from CE- and E₂-treated rats with the following exception: E₂ - 4-20 days all the groups, and CE - 24 days, preoptic, cortex and plasma. Note also that plasma LH values in CE-treated rats were significantly reduced ($p < 0.05$) from 2 h to 24 days when compared with DMSO-treated rats, and that plasma LH values of E₂-treated rats were only different between 2 h and 4 days compared to DMSO-treated rats.

Fig. 4.

Effects of CE and E₂ on body weight in pubertal female rats. Animals received a single intravenous injection of CE at a dose of 2.56 (□), 12.8 (■), 25.6 (▲) or 51.2 μmol/kg (●), or a single injection of E₂ (25.6 μmol/kg; △) or DMSO vehicle (1 ml/kg; ○) alone. Values are mean ± SE of 5 rats. Data for each rat were normalized to its initial body weight. All the values of CE-treated rats were significantly different ($p < 0.01$) from DMSO-treated groups at the same sampling time between days 4 and 28. The values in E₂-treated rats were significantly different ($p < 0.01$) from DMSO-treated rats at the same sampling period between days 4-12. The growth curve of E₂-treated rats was also different ($p < 0.005$) from CE (12.8-51.2 μmol/kg)-treated rats.

Fig. 5.

Comparison of the action of CE and E₂ on body weight in OVEX rats. Animals received a single intravenous injection of CE (7.7 μmol/kg; □), E₂ (7.7 μmol/kg; ○) or DMSO vehicle (1 ml/kg; Δ) alone. Data are mean + SE of 4-6 rats. Values of each rat were normalized to its initial body weight. The values of CE-treated rats were significantly different ($p < 0.01$) from DMSO-treated rats at days 1-24 and from E₂-treated rats at days 2-24. The E₂-treated rats had reduced ($p < 0.05$) body weights until day 16 compared with DMSO-treated rats.