Potential Estrogenic Properties of 17β-Hydroxy-ethylimine Estradiol Derivative Targeted to Menopause Stage

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Background/Aim: Hormone replacement therapy during menopause increases the risk of thromboembolic diseases and cancer, so safety alternative therapeutic strategies are needed. 17β-Aminoestrogens are a synthetic estrogens group that possess mild anticoagulant activity that contrasts with the pro-coagulant effects showed by estradiol’s (E2) in rodents. Being considered an alternative to conventional hormone replacement therapy during menopause without thrombogenic risks producing. The present study aimed to determine the estrogenic profile and anxiolytic activity of 17β-[hydroxy-ethylimine]-1,3,5(10)-estratrien-3-ol (IE2), a related compound unknown until now. Methods: IE2 was assessed in immature rats by uterotrophic assay administering IE2, E2, or vehicle. In ovariectomized adult Wistar rats (Ovx) to facilitating the lordotic behavior compared with E2, estradiol benzoate, or vehicle. The effect of IE2 on anxiety was estimated in Ovx animals treated with IE2, E2, or vehicle group and evaluated in the elevated plus-maze model. Results and conclusion: IE2 produced an uterotrophic effect, lordotic behavior, and anxiolytic effect in a dose-dependent manner, similar to E2. IE2 depicted estrogenicity, indicating potential clinical use as hormone replacement therapy during menopause.

Key words 17β-aminoestrogen; estradiol; anxiety; lordosis behavior

INTRODUCTION

Sex steroid hormones are involved in the differentiation and maintenance of sexual characteristics and reproduction. The ovaries cyclically secrete estrogens from puberty to menopause; they profoundly influence women’s physiology and behavior. In perimenopause, estrogens deficiency produces physiological changes such as hot flashes, night sweats, irregular periods, insomnia, the decay of sexual motivation, also psychological changes as symptoms of anxiety, depression, and alterations in cognitive functions.1 Anxiety disorders have a population incidence of 5% that can increase up to 10% in women over 40 and are more severe during perimenopause and menopausal periods.2 Estrogen levels reduction at this stage is an indicator of the participation of their modulation in the affective state. Hormone replacement therapy (HRT) with conjugated equine estrogens, ethinyl estradiol, and estradiol derivatives are used to manage peri- and menopause symptoms.3 However, many clinical studies described the high risk of adverse effects associated with its use, such as thromboembolic events and cancer.4 Thus, taking into account them, it is recommended to prescribe HRT at the minimum effective dose and for the shortest time possible under closed supervision.5 Moreover, lifespan has increased in the human population, and the menopausal stage is a long period for most women to cope with the deficiency.

In the search for alternative synthetic estrogens without producing thromboembolic risk, our group has reported the biological characterization of the synthetic estrogens 17β-aminoestrogens. These compounds possess anticoagulant effects, which contrast with the pro-coagulant effects of 17β-estradiol (E2) and other estrogen derivatives used in HRT. They behave as estrogenic partial agonists of E2, increasing uterine weight, activating estrogen receptor transcription, and decreasing luteinizing hormone. Also, they induce progesterone receptors synthesis in the anterior pituitary gland and stimulate sexual behavior (lordosis) in the ovariectomized (Ovx) rat.7 As part of the characterization of the activity profile of the 17β-aminoestrogens, the anxiolytic, anti-depressant, and mnemonic properties have been described in the Ovx rat.8 The 17β-aminoestrogens low estrogenic properties in peripheral tissues, coupled to their enhanced activity in the central nervous system (CNS) without producing prothrombotic effects, could be an alternative HRT directed to menopausal women.

The objective of the present work was the evaluation of the estrogenic and anxiolytic effects of an imino estradiol derivative, 17β-[hydroxy-ethylimine]-1,3,5(10)-estratrien-3-ol (IE2) (Fig. 1). A compound with a closed structural relation to 17β-aminoestrogens that has not been biologically evaluated until now.

Fig. 1. Chemical Structure of IE2

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MATERIALS AND METHODS

Reagents  17β-Estradiol (E$_2$: 1,3,5(10)-estratrien-3,17β-dirol), 17β-estradiol benzoate (EB; 1,3,5(10)-estratrien-3,17β-diol 3-benzoate), and progesterone (P; Preg-n-4-ene-3,20-diona) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). 17β-[Hydroxi, ethylime]-1,3,5(10)-estratrien-3-ol (IE$_2$, Fig. 1), was prepared from estrone according to the Im scher method. Chemical purity was established by chromatographic (HPLC, TLC) and spectral (IR/NMR/MS) techniques.

Animals  The study was carried out at the Laboratory of Endocrine Pharmacology, Department of Pharmacology, Faculty of Medicine, National Autonomous University of Mexico (UNAM), Mexico City, and approved by the Local Animal Ethics Committee. All experimental studies were conducted following the Mexican National Protection Laws of Animal Welfare (NOM-062-Z00-1999). Wistar immature female 21 d old (30–40 g), adult female, and male eight-nine weeks old (200–250 g) rats from our animal breeding facilities were used. In the beginning, groups of five rats were housed in standard local acrylic cages with autoclaved wood shaving bedding (90–100 g/cage). The immature female rats for the uterotrophic assay were kept in a room with a 12–12 light–dark cycle (lights on from 8:00 to 20:00). Adult male and female Wistar rats evaluated for anxiety and facilitation of sexual behavior experiments were kept in a reversed 12–12 h light–dark cycle (lights off from 8:00 to 20:00). All animals were maintained with constant temperature (20–22°C), humidity range of 50–10% fed with a commercial diet (Nutricia, Purina), and tap water ad libitum. On completion of each experiment, rats were euthanized with an overdose of chloral hydrate anesthesia.

Evaluation of Uterotrophic Effect  Immature animals were distributed among groups according to a balanced design based on body weight. Groups (n = 8–10) were randomly assigned to treatment groups. Animals were subcutaneously (s.c.) injected once a day for 3 consecutive days with: E$_2$ (1, 10, 50, 100, 250, 500 µg/kg) or IE$_2$ (1.3, 6.7, 13, 55, 110 µg/kg), the control group received the vehicle (10 mL/kg) only. On the fourth day, the animals were weighed, and the uterotrophic activity induced by the tested compounds was evaluated by the software Origin 8.0 (OriginLab Corporation, Northampton, MA, U.S.A.).

Data Analysis  The results were expressed in means ± standard error of the mean (S.E.M.). Percent differences with respect to the vehicle group were obtained by the relation: [Treated group (100)/control group] = $\frac{\text{max E}_2}{\text{max IE}_2}$, where min is the base response, max correspond to the maximal response, $X$ represents the log dose, $K$ is ED$_{50}$ and $n$ is the Hill coefficient. The ED$_{50}$ and the maximum response value (E$_{max}$) were obtained by the software Origin 8.0 (OriginLab Corporation, Northampton, MA, U.S.A.). Statistical significance among the different treated groups was estimated by a one-way ANOVA test. The significance of the differences between control (vehicle) and treated groups was assessed by the Post Hoc test (Tukey, Dunn’s, or Dunnet’s methods) as required. p < 0.05 was considered as the limit for statistical significance. The analysis was performed using the Sigma Plot software 11.0 (Jandel Corporation).
RESULTS

IE₂ Uterotrophic Effect  IE₂ produced uterotrophic effects in immature rats. Figure 2 shows a significant dose-dependent increase ($p<0.05$ vs. vehicle) in the uterine weight of prepubertal rats treated with different doses of IE₂ and E₂. The related to E₂ (9.6 ± 0.8 µg/kg) produced a significant dose-dependent effect to E₂ (103 ± 5 µg/kg) and IE₂ (110 µg/kg) respectively, which were more potent in both Uww and Udw with respect to the E₂ (20 ± 1.5 µg/kg) related to E₂ (9.6 ± 2.3 and 9.4 ± 2.0 µg/kg, respectively), which demonstrated lower potency than E₂.

Table 1. Uterotropic Effect ED₅₀ and Emax of E₂ and IE₂ of Prepubertal Wistar Rats

|         | Uww | Udw |
|---------|-----|-----|
| Vehicle | 22 ± 0.9 | 4 ± 0.8 |
| E₂      | 103 ± 5 | 20 ± 1 |
| IE₂     | 138 ± 17 | 42 ± 6 |

The results obtained by this model indicate that IE₂ exhibits estrogenic activity with higher efficacy than E₂, which had the lowest efficacy of 53%. The LQED₅₀ of EB was 10.9 µg/kg, showing to be more potent than E₂ and IE₂ (20.9 and 128.8 µg/kg, respectively). The results obtained by this model indicate that IE₂ exhibits an estrogenic activity with higher efficacy than E₂ to induce sexual receptivity in Ovx rats; however, with lower potency.

IE₂ Evaluation in the EPM  The EPM test was carried out to assess the anxiolytic effect of IE₂ compared to that produced by E₂ in Ovx rats. The groups treated with E₂ (25 ± 1.7 µg/kg), IE₂ (55 ± 110 µg/kg) maintained a time spent into open arms 38, 43 and 45% respectively, which were significantly longer than those observed in the control group (V = 46%, Fig. 4A). Consequently, the time spent by the Ovx rats different groups into closed arms was as follows: E₂ 30%, IE₂ (55) 35% and IE₂ (110 µg/kg) 28%, contrasting with the control group that remained 46% of the time in closed arms (Fig. 4B). The ANOVA analysis showed significant differences between the control group and the two IE₂ groups (55 and 110 µg/kg) (25, 20.9 and 128.8 µg/kg, respectively), which had the lowest efficacy of 53%. The LQED₅₀ of EB was 10.9 µg/kg, showing to be more potent than E₂ and IE₂ (20.9 and 128.8 µg/kg, respectively). The results obtained by this model indicate that IE₂ exhibits an estrogenic activity with higher efficacy than E₂, which had the lowest efficacy of 53%.

DISCUSSION

The present study shows that IE₂ produced high estrogenic activity in Ovx rats using two classical paradigms for estrogenic activity evaluation: the uterotrophic assay, as well as an inducer of the sexual behavior of the rat. IE₂ in the uterus of immature mice induced a dose-dependent increase in uterine weight similar to that exerted by E₂, a phenomenon that is consistent with other reports. Its uterotrophic potency evalu-
ated by the wet uterine weight (Uww) was higher than that exhibited by E₂. Probably IE₂, in the same way as E₂, will also increase the uterine tissue (endometrium, myometrium, luminal epithelium, etc.), in all likelihood acting through the activation of estrogen receptors (ERs) by stimulating the transcription of specific genes in reproductive function. The higher uterotrophic potency of IE₂ contrasts with the effects observed in the Ovx rats treated with 17β-aminoestrogens, which have a very low uterotrophic efficacy and potency.¹⁴ These compounds are modified estrogens where the C17 has a -N-[CH₂]ₘ-OH group, causing the estrogenic activity to decrease as the number of methylenes increases. The structural change significantly impacts their estrogenic potency, being up to 500 times lower than that of E₂ when n = 5 in uterotrophic trials in young Ovx rats and immature rats and mice.¹⁴ The low estrogenic activity is related to the lower affinity of 17β-aminoestrogens for the estrogen intracellular receptors ERα and ERβ concerning E₂.¹⁵ The chemical structure of IE₂ contains the group R = N-[CH₂]₂-OH, where R = C17 of the steroidal ring similar to that of E₂, which favoring uterotrophic efficacy and potency and could be indicating a higher affinity for IE₂ intracellular ERs related to that exhibited by the 17β-aminoestrogens.

On the other hand, IE₂ produces effects similar to those induced by E₂ at the CNS level. The administration of E₂ and IE₂ sequentially with P induced a lordotic response in

Table 2. LQ Eₘₐₓ, LQED₅₀ of E₂, EB, IE₂ in the Ovx Rats

| Group | LQ Eₘₐₓ (LQ ± S.E.M.) | LQ ED₅₀ (µg/kg) | Relative effect |
|-------|-----------------------|----------------|-----------------|
| Vehicle | 0                     | 0               | 100             |
| E₂    | 53 ± 10               | 20.9 ± 1.3      | 180             |
| EB    | 92 ± 11               | 10.9 ± 1.2      | 165             |
| IE₂   | 86 ± 12               | 128.8 ± 6.0     | 165             |

LQ Eₘₐₓ = mean maximum LQ ± S.E.M.; LQED₅₀ = effective dose to produce LQ₅₀. Relative effect to E₂ = [LQ Eₘₐₓ EB or IE₂]100/(LQ Eₘₐₓ E₂).

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Fig. 3. Dose–Response Curves of the Effect of IE₂ in the Sexual Behavior of Female Rats

Different groups of Ovx rats were treated with a single administration of different doses of IE₂, as controls estradiol benzoate (EB) estradiol (E₂) and the vehicle included as a starting point in the graph. Their efficacy order was EB > IE₂ > E₂. Meanwhile their potency order was: EB > E₂ > IE₂. Each point represents the mean ± standard error of 8–10 animals per group.

Fig. 4. The Anxiolytic Effect of IE₂ in the EPM

The values represent the mean ± standard error of the percentages of time that the different groups of animals spent in open arms (a), or closed arms (B) and the entries number into the open arms (C) and closed arms (D) for 5 min. Each point represents the mean ± standard error of 8–10 animals per group. *p < 0.05 vs. control.
young Ovx rats in a dose-dependent manner. The maximum response of IE₂ (LQ = 86%) was more effective than E₂ (LQ = 53%) although, its relative potency was lower (ED₅₀: IE₂ 129 and E₂ 21 μg/kg). These results show that IE₂ acts on sexual receptivity in young Ovx rats in a similar manner to that induced by E₂. Treatment with E₂ and P modulates the neural activity and induces preceptive and receptive behaviors in Ovx rats. However, the control of lordotic reflex in the rat is a complex issue. Several systems and events are coordinated at the hypothalamic level in the ventromedial nucleus (VMH), medial preoptic nucleus (MPN), and mesencephalic level periaqueductal gray (PAG) matter. Which modulate and integrate hormonal, neurochemical, and sensory behavioral information. Many neurotransmitter systems (serotonin, dopamine, norepinephrine, γ-aminobutyric acid (GABA), etc.) and neuropeptides located in the brain areas are involved in the control of sexual receptivity. The receptive sexual behavior (lordosis), induced by E₂, in the rat is related to the increased progesterone receptors synthesis by genomic events due to the gene transcription stimulation, which increases the P effectiveness. However, non-genomic effects occur at the membrane level, such as the ion flow and neurosteroid action. The 5α-pregn-3α-ol-20-one (3α, 5α-THP; also known as allopregnanolone) in the ventral tegmental area (VTA); is de novo synthesized from cholesterol in both the CNS and the peripheral system from its precursor P, which concentration is independent of the endocrine gland secretion. Neurosteroids act in the brain through ionotropic receptors, being positive allosteric modulators of GABAₐ receptors. In VTA, 3α, 5α-THP activates the GABAₐ receptor and prolongs the Cl⁻ channels opening, which leads to an increase in the duration of inhibitory postsynaptic potential.

The GABAergic system, as the primary inhibitor of neuronal excitability of vertebrates, is involved in the regulation of fear and anxiety. The anxiolytic effects of E₂ in female ovariectomized rodents have been demonstrated. Ovariectomy decreased E₂ and P levels. In the brain of Ovx animals, GABAₐ levels, along with the number of GABAergic receptors, is decreased. The Ovx animals display anxiety, and depressive-like behaviors, which can be reverted by the sequential administration of E₂ and P, or allopregnanolone.

IE₂ produced anxiolytic effects similar to those observed in Ovx rats treated with E₂ obtained through the EPM paradigm. Animals treated with IE₂ spent more time in the open arms of the EPM maze related to the control group, and its effects were very similar to those observed in animals treated with E₂. Under normal conditions, rodents enter the closed arms and avoid the open arms of the EPM. Anxiolytics agents such as diazepam, increase the rodent’s permanence time in open arms, which is considered an anxiolytic-like effect. Some hormonal agents such as P, 5α-THP, and estrogens like E₂ are anxiolytic in the EPM.

On the other hand, also is the consideration estrogens influence on the mono-amnergic neurotransmitter systems (serotonin, norepinephrine, and dopamine) involved in the pathophysiology of affective disorders, such as depression. Since E₂ modulates biosynthesis, transport, and metabolism, determining the number of receptors in their activity area. The IE₂ anti-anxiety properties mechanism needs to be studied to increase the evidence on its estradiol-like anti-anxiety effects.

CONCLUSION
The results showed here, particularly the IE₂ induction of lordotic behavior and its anti-anxiety properties show its capability to reach CNS exerting properties similar to that shown by E₂. Our results open the way for further research on the effects of IE₂ or steroidal imines substituted at the C17 as anxiolytic agents. Furthermore, it is no prothrombic effects, cardiovascular effects, or cellular promotion of hormone-dependent malignancies would also have to be considered for future studies. They encourage abor toxico logical studies, biological targets, and mechanisms of action at the CNS level to know their potential clinical use associated with anxiety drugs or depression disorders during the menopause period.

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Conflict of Interest The authors declare no conflict of interest.

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