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Estrogens and menopause: pharmacology of conjugated equine estrogens and their potential role in the prevention of neurodegenerative diseases such as Alzheimer's☆

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Abstract

Menopause marks the start of a new phase in a woman's life that is associated with a decrease in circulating estrogen levels. Although the average age of women has increased from 50 to nearly 85 years, the average age at menopause has remained essentially constant at 50 years. Thus, women now spend nearly a third of their lives in an estrogen deficient state. This normal aging process in women is associated with increasing health problems such as osteoporosis, cardiovascular disease, neurodegenerative diseases, and cancer. Estrogen replacement therapy (ERT) has been shown to play an important beneficial role in the health and well being of postmenopausal women. Several estrogen preparations are available and among these conjugated equine estrogens (CEE) are most frequently used. The drug CEE, is a complex *natural* urinary extract of pregnant mare's urine and contains at least 10 estrogens in their sulfate ester form and these are the ring B saturated estrogens: estrone (E_1), 17β-estradiol (17β-E_2), 17α-estradiol (17α-E_2), and the ring B unsaturated estrogens equilin (Eq), 17β-dihydroequilin (17β-Eq), 17α-dihydroequilin (17α-Eq), equilenin (Eqn), 17β-dihydroequilenin (17β-Eqn), 17α-dihydroequilenin (17α-Eqn), and Δ^8-estrone (Δ^8-E_1). All of these estrogens in their unconjugated form are biologically active and can interact with recombinant human estrogen receptor α (ERα) and β (ERβ) with 17β-estradiol and 17β-dihydroequilin having the highest affinity for both receptors. A number of the ring B unsaturated estrogens had nearly twofold higher affinity for the ERβ. The pharmacokinetics of these estrogens in postmenopausal women indicate that the unconjugated estrogens compared to their sulfated forms are cleared more rapidly. The 17-keto estrogens are metabolized to the more potent 17β-reduced products which are cleared at a slower rate. In postmenopausal women, the extent of 17β-activation is much higher with the ring B unsaturated estrogens than with ring B saturated estrogens. Oxidized LDL and oxidative stress are thought to contribute to both atherosclerosis and neurodegenerative disorders. Neurons in particular are at a high risk from damage resulting from oxidative stress. In vivo and in vitro studies indicate that the oxidation of LDL isolated from postmenopausal women was inhibited differently by various estrogens and other antioxidants. The unique ring B unsaturated estrogens were the most potent while the red wine component t-resveratrol was the least potent.

Studies were designed to explore the cellular and molecular mechanisms that may be involved in the neuroprotective effects of CEE components. The data indicate that the neurotoxic effects of oxidized LDL and glutamate can be inhibited by various estrogens, with the ring B unsaturated estrogens being the most active. These effects are involved in the inhibition of DNA fragmentation and up-regulation of anti-apoptotic protein Bcl-2 and down-regulation of pro-apoptotic protein Bax. These combined data suggest that some of the neuroprotective benefits associated with long-term estrogen therapy may occur by the above mechanism(s). Because estrogens such as the Δ^8-estrogens are relatively less feminizing than the classical estrogen 17β-estradiol, they may be important in the development of more neuro-specific estrogens that will be useful in the prevention of neurodegenerative diseases, such as Alzheimer's and Parkinson disease, in both men and women.

Keywords: Estrogens and menopause; Conjugated equine estrogens; Neurodegenerative diseases

1. Introduction

Menopause marks the start of a new phase in a woman's life that is associated with a decrease in circulating estrogen levels [1], cessation of ovarian function and menstrual bleeding. Natural menopause is a physiologic process that occurs in healthy women at a median age between 50 and 55 years [1]. During the past century, the general world

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population and the elderly population, has been increasing at a rapid rate [2]. Projections indicate that approximately 124 million people are expected to be over 80 years plus by the year 2020. The majority of this elderly population is expected to be postmenopausal women [2]. The average life expectancy of women in the next millennium is expected to be over 80 years and thus women will spend over one-third of their lives in an estrogen deficient state. This normal aging process in women is associated with increasing health problems such as osteoporosis, coronary heart disease, neurodegenerative diseases such as Alzheimer's, cancer, vulvo-vaginal atrophy, hot flashes and depression. A number of therapeutic agents are available to prevent or delay the onset or progression of some of the above disorders. Among these, estrogen replacement therapy (ERT) and hormone replacement therapy (HRT: estrogen plus progestin) are most frequently used. Although there are several estrogen preparations in the market, the most prescribed natural estrogen for the past 60 years is conjugated equine estrogen (CEE; Premarin, Wyeth, Philadelphia, USA). This drug contains at least 10 estrogens in their sulfate ester form and these are the ring B saturated estrogens: estrone (E_1), 17β-estradiol (17β-E_2), 17α-estradiol (17α-E_2), and the ring B unsaturated estrogens equilin (Eq), 17β-dihydroequilin (17β-Eq), 17α-dihydroequilin (17α-Eq), equilenin (Eqn), 17β-dihydroequilenin (17β-Eqn), 17α-dihydroequilenin (17α-Eqn), and Δ^8-estrone (Δ^8-E_1). The structures of these estrogens are shown in Fig. 1. This paper deals with the pharmacology of the equine estrogens and their potential role in the prevention of neurodegenerative diseases such as Alzheimer's disease (AD).

1.1. Absorption

For an estrogen to exert its biological effects, it must first be absorbed, then reach and interact with its receptors in target tissues. The rate at which these events occur depends on the route of administration and these have been extensively reviewed [3] and will be briefly discussed in this article.

The oral route of administration is the most frequently used method and is the preferred route by a majority of postmenopausal women. Oral estrogen by virtue of the first pass effect results in an increase in angiotensinogen, sex hormone binding globulin (SHBG) corticosteroid binding globulin (CBG), high density lipoprotein cholesterol (HDLc), triglycerides, clotting factors, a decrease in low density lipoprotein cholesterol (LDLc) and total cholesterol levels. Although these metabolic changes can have important clinical implications particularly on cardiovascular disease in postmenopausal women [4,5], some of the beneficial effects of HRT towards the prevention of cardiovascular disease in postmenopausal women have been questioned by the results of the Women's Health Initiative Study (WHI [6]). The study was stopped early as the investigators felt that HRT use resulted in more harm than benefit. This randomized controlled trial (RCT) was unfortunately carried

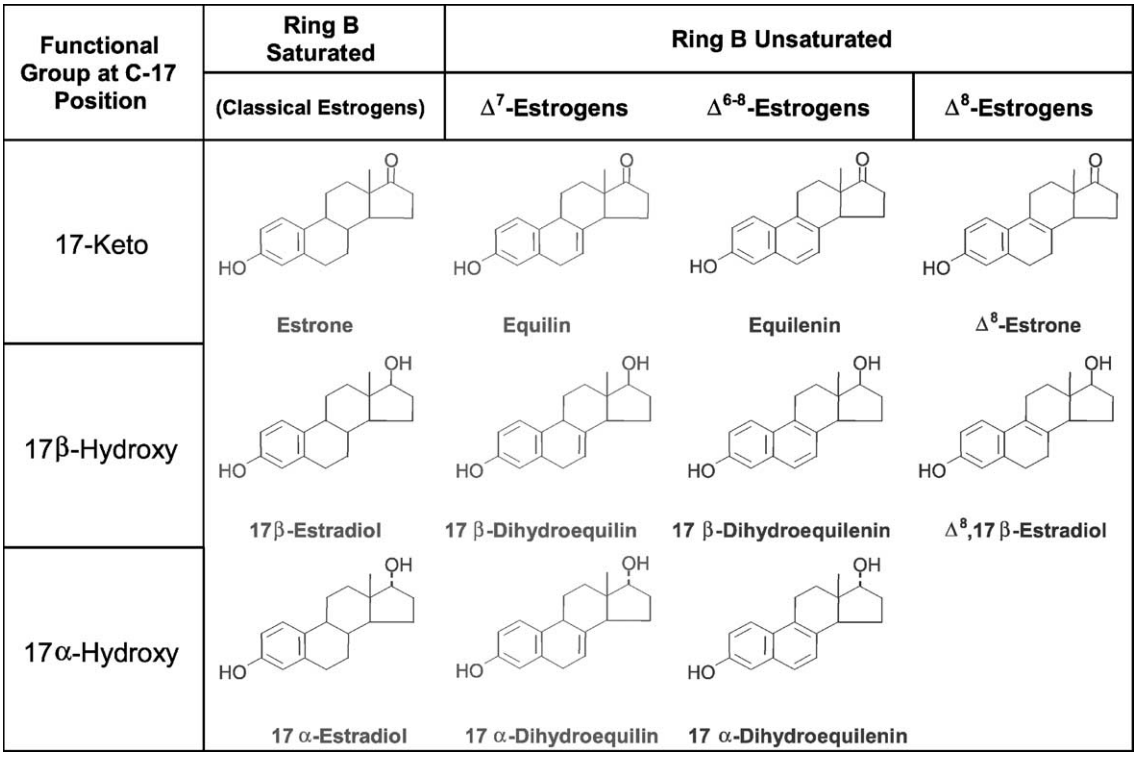

Fig. 1. Structure of equine estrogens. With the exception of Δ^8,17β-estradiol, all estrogens in their sulfated form are components present in the drug conjugated equine estrogens ([49] with permission).

out in women whose average age was 63.3 years and nearly 70% of the women enrolled in the study were overweight and of these, half were obese (body mass index ≥30), 36% treated for hypertension, and only 50% had never smoked. Although the WHI–RCT was considered to be a primary prevention trial, the fact that 45.2% of the women were in their 60s and 21.5% in their 70s, many of the age-related factors that increase the risk of cardiovascular disease in postmenopausal women had already been initiated. Therefore, the WHI–RCT is not a true primary prevention study of healthy postmenopausal women [7]. Interestingly, the ERT arm of WHI is still ongoing (>6 years) suggesting that CEE alone may not be associated with any significant increase in the risk of breast cancer, stroke, and cardiovascular disease.

Although all oral estrogens undergo first pass effect, the amounts bio-available are quite variable and this is reflected in their potency. Thus on weight basis, CEE are up to three times more potent than micronized estradiol and piperazine estrone sulfate in inducing hepatic synthesis of SHBG, CBG and angiotensinogen [8]. Higher levels of SHBG will result in a lower plasma level of the physiologically free form of estrogen available for biological action.

1.2. Metabolism-interconversions between estrogens under steady state conditions

In postmenopausal women, estrone, Δ^8-estrone and equilin are metabolized to circulating 17β-estradiol, Δ^8-17β-estradiol, and 17β-dihydroequilin, respectively [9–12]. The precise conversion ratios of some of these metabolites have been determined [13,14] and the results indicate that ring B unsaturated estrogen components of CEE are more extensively metabolized than the classical estrogens E_1 and 17β-E_2. The interconversions further indicate that the oxidative pathway is generally preferred compared to the reductive pathway [15], however, the extent of 17β-reduction of ring B unsaturated estrogens Eq and Δ^8-E_1 is several fold greater than with estrone [13,14,16]. Since 17β-reduced products are considered to be the active metabolites, metabolic differences between various types of estrogens may influence the overall biological and clinical activity.

1.3. Metabolic clearance rate (MCR) of conjugated and unconjugated equine estrogens

The MCR of an estrogen is defined as the volume of plasma (blood) from which the estrogen is totally and irreversibly cleared in unit time (liters per day or liters per day/m^2) and provides information regarding the overall in vivo metabolic fate of the estrogen. The MCR of some equine estrogens are given in Table 1. The MCRs of unconjugated Eq, 17β-Eq, E_1, 17β-E_2 are several fold higher than those of their corresponding sulfate forms, indicating that the sulfate conjugated estrogens are cleared from the circulation at a much slower rate [3,8,10–14,16,17]. These observations support the hypothesis that sulfate conjugated estrogens in circulation act as reservoirs from which the biologically active (unconjugated) forms are continuously being produced. The data in Table 1 further indicates that ring B unsaturated estrogen sulfates are cleared faster than the ring B saturated estrogen sulfates. Similarly, the MCRs of the 17β-reduced estrogens are approximately two times lower than their corresponding 17-keto forms. This is most likely due to the higher binding affinity of the 17β-reduced forms for SHBG.

Table 1
Metabolic clearance rate of estrogens

Estrogen	MCR (per day/m^2) ± S.E.M.	Reference
Estrone sulfate	80 ± 10[a]; 105 ± 20	[9,10]
Equilin sulfate	176 ± 44[a]; 170 ± 18	[11,13]
17β-Dihydroequilin sulfate	376 ± 53[a]; 460 ± 60	[12,14]
Estrone	1050 ± 70	[15,17]
17β-Estradiol	580 ± 30	[15,17]
Equilin	2641[a]	[11]
17β-Dihydroequilin	1252 ± 103[a]	[12]
Δ^8-Estrone	1711 ± 252[a]	[16]

[a] MCR measured by the single injection technique.

1.4. Differential interaction of equine estrogens with recombinant human estrogen receptors α and β

The basic genomic mechanism of steroid hormone action has been known for more than three decades and detailed reviews are available [1,3,18–21]. The human estrogen receptor (ER) exists as two subtypes, ERα and ERβ, which differ in the N-terminal transactivation domain and in the C-terminal ligand binding domain. In the present investigation, the binding affinities of the 10 equine estrogens shown in Fig. 1 and Δ^8,17β-estradiol, a major in vivo metabolite of Δ^8-E_1 in postmenopausal women [16] for recombinant human estrogen receptors ERα and ERβ, were determined. Saturation binding analysis of human ERα and ERβ protein revealed a single population of high affinity binding sites for [^{3}H]17β-E_2 with a kd of 0.06 nM for ERα and 0.1 nM for ERβ (Fig. 2). All of the remaining 10 estrogens competed with [^{3}H]17β-E_2 for binding to both ERα and ERβ with varying affinities. Examples of typical competition curves obtained are shown in Fig. 3. From the slopes, the IC_{50} values were calculated and these were used to calculate the relative binding affinities (RBA); RBA for 17β-E_2 for both receptors was set at 100 and the RBA of all estrogens tested are given in Table 2. The RBA of 17β-Eq for both receptor subtypes was similar to that of 17β-E_2 (Table 2), while those of the remaining estrogens were lower but were in general higher for ERβ. Compared to 17β-E_2, the RBA's of ring B unsaturated estrogens: Eq, Eqn, Δ^8-E_1, and 17α-Eqn for ERα and ERβ were five to eightfold and 2–4 times lower respectively (Table 2). However, these four estrogens have two to four times greater affinity for ERβ than for ERα. The order of competition with ERα was: 17β-Eq > 17β-E_2 > 17β-Eqn = Δ^8,17β-E_2 > 17α-Eq > E_1 ≥ 17α-Eqn > Δ^8-E_1

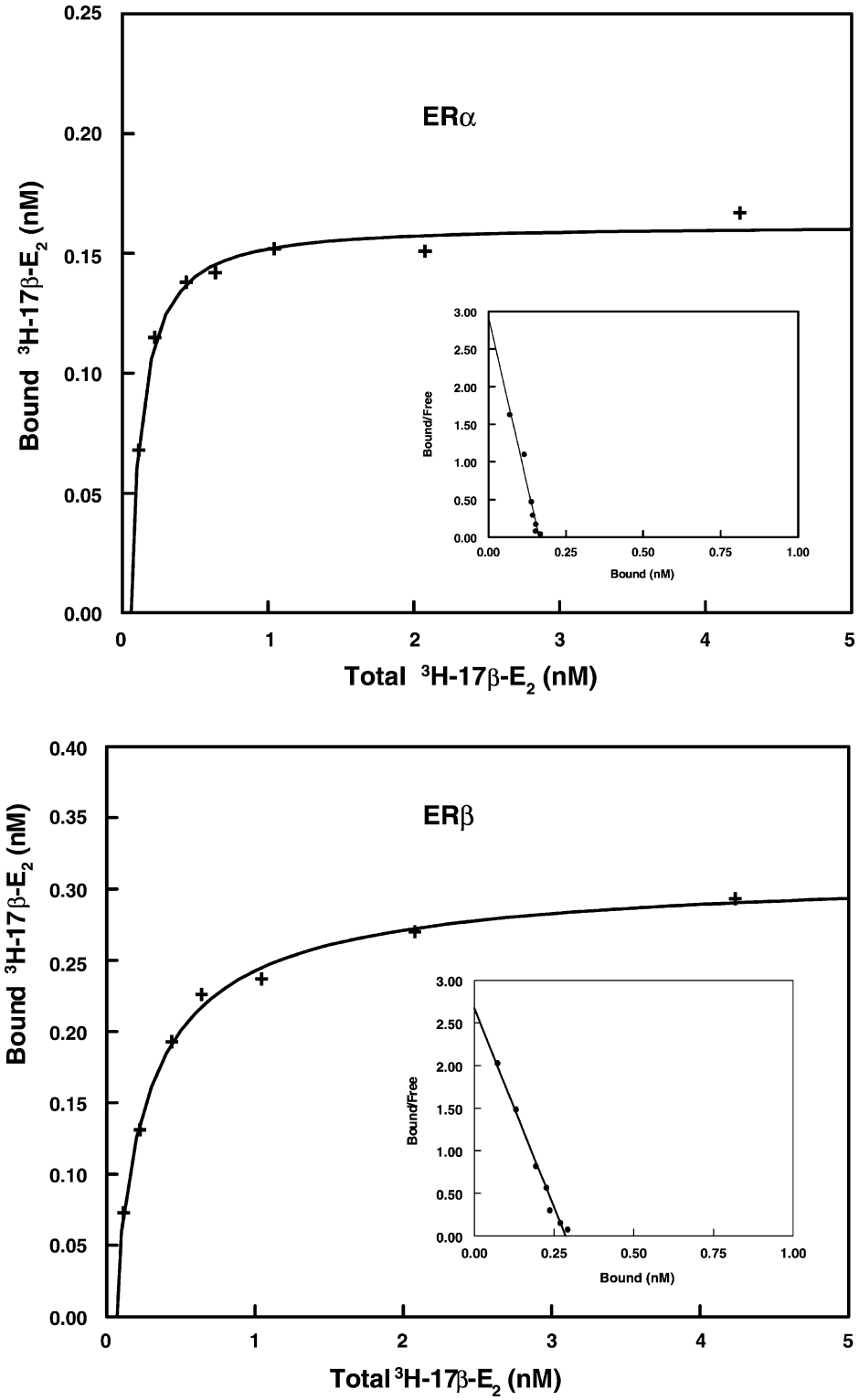

Fig. 2. Specific binding of [^{3}H]17β-estradiol with ERα and ERβ. (Inset) Scatchard plots of the data.

$\geq$ 17α-E_2 > Eqn > Eq and for ERβ 17β-Eq > 17β-E_2 > 17β-Eqn > Δ^8,17β-E_2 > E_1 > 17α-Eqn = Eq > 17α-E_2 > 17α-Eq = Δ^8-E_1 > Eqn.

These results further indicate that both ERα and ERβ have greater affinity for the 17β-stereoisomers than the corresponding 17α-isomers. This is in keeping with previous binding affinities reported for 17β-E_2 and 17α-E_2 for ERα and ERβ [22]. Results also indicate that all equine estrogens bind with human ERα and ERβ with high affinity. Although the interaction of ring B unsaturated estrogens with crude

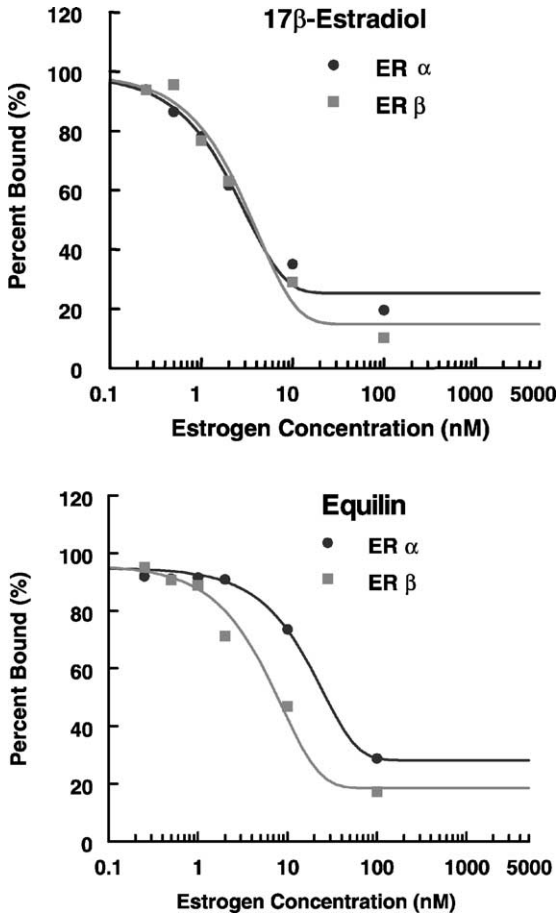

Fig. 3. Competition by some equine estrogens for [^{3}H]17β-E_2 with recombinant human ERα and ERβ. The receptors were incubated with 2 nM [^{3}H]17β-E_2 and various concentrations of unlabeled competitors. Incubations were carried out for 18 h at 4C, bound and free forms were separated by using hydroxlapatite.

Table 2
Relative binding affinities of various equine estrogens for recombinant human estrogen receptors α and β

Estrogens	RBA[a]		
	ERα	ERβ	ERα/ERβ
17β-Estradiol	100	100	1.00
17β-Dihydroequilin	113	108	1.05
17β-Dihydroequilenin	68	90	0.75
Δ^8,17β-Estradiol	68	72	0.94
17α-Dihydroequilin	42	32	1.30
Estrone	26	52	0.50
17α-Dihydroequilenin	20	49	0.40
Δ^8-Estrone	19	32	0.60
17α-Estradiol	19	42	0.45
Equilenin	15	29	0.50
Equilin	13	49	0.26

The IC_{50} of 17β-E_2 was 3.4 nM for ERα and 3.8 nM for ERβ. Concentration of ^{3}H, 17β-estradiol 2 nM; ERα and ERβ 1 nM kd; 17β-E_2 for ERα 0.06 nM kD; 17β-E_2 for ERβ 0.10 nM.

[a] $\text{RBA} = \frac{IC_{50} \text{ of } 17\beta\text{-estradiol}}{IC_{50} \text{ of competitor}} \times 100$.

human endometrial cytosolic and nuclear receptors has been previously described [23], to our knowledge, this is the first study where the interaction of the ring B unsaturated equine estrogens with human ERβ-protein has been reported.

The observation that some of these estrogens have greater affinity to ERβ will facilitate development of specific ERβ ligands that may not only help understand the differences in the biological functions of ERβ and ERα but may be of therapeutic use in tissues where ERβ predominates, for example, in specific areas of the brain and colon.

1.5. Antioxidant properties of equine estrogens and their potential role in atherosclerosis and Alzheimer's disease

Several lines of evidence suggest that oxidatively modified low density lipoprotein (LDL) plays an important role in the etiology of atherosclerosis and neurodegenerative diseases such as Alzheimer's disease [24–31]. Oxidized LDL (oLDL) is formed in vivo, perhaps by a free radical-based mechanism, and is more atherogenic and neurotoxic than native LDL [24–31]. Lipoproteins have been detected in the cerebral spinal fluid [32] and LDL is transported into the brain by a carrier protein [33,34]. In the brain, LDL is exposed to a highly oxygenated and lipid-enriched environment, making it susceptible to free radical-mediated lipid peroxidation that can result in the formation of oLDL [35]. Based on these observations, we hypothesize that oLDL may be involved in both atherosclerosis and neurodegenerative diseases and that equine estrogen can inhibit both its formation and its cytotoxic effects. To further study this, the following investigations were carried out.

In the first study, we compared the antioxidant potency of a number of equine estrogens: E_1, 17β-E_2, 17α-E_2, Eq, 17β-Eq, 17α-Eq, Eqn, 17β-Eqn, 17α-Eqn, Δ^8-E_1, Δ^8,17β-E_2, with the antioxidant activity of trolox, a synthetic water soluble analog of Vitamin E, probucol (4,4′-[(1-methylethylidene)bis(thio)bis2,6-bis(1-1dimethyl-gl-ethyl)] phenol, a drug used for lowering cholesterol, and the two red wine components quercetin and t-resveratrol. In this in vitro study, LDL was isolated from healthy postmenopausal women who were not on any hormone therapy. The kinetics of LDL oxidation were measured by continuously monitoring the formation of conjugated dienes followed by determination of the lag time [36]. The data indicated that all of the compounds tested inhibited the oxidation of LDL (prolongation of lag time) in a dose-dependent manner. The data also showed that at the lowest concentration (0.15 nmol) of estrogen tested, the lag time increased by 33–209% over the control value. At the lowest dose of quercetin and trolox, lag time was prolonged by 74 and 25%, respectively. At low concentrations, t-resveratrol and trolox were ineffective [36]. The minimum dose (nmoles) required to double the lag time from control lag time of 57 ± 2 min was 0.47 for 17β-Eqn, 17α-Eqn, Δ^8-E_1; 0.6–0.7 for Δ^8,17β-E_2, Eqn and quercetin; 0.9 for 17β-Eq and 17αEq; 1.3 for Eq, E_1, 17β-E_2, 17α-E_2; 1.4 for trolox; 1.9

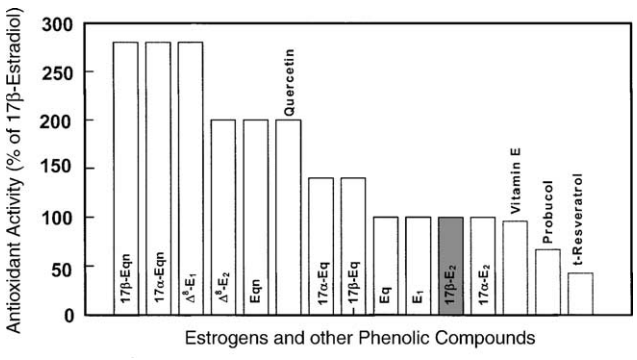

Fig. 4. Antioxidant activity of various estrogens and other phenolic compounds expressed as a percentage of 17β-estradiol activity ([36] with permission).

for probucol and 3.0 for t-resveratrol. Since 17β-E_2 is the most potent (uterotropic) endogenous estrogen, the antioxidant activity of the various compounds was compared to this estrogen. The data expressed as percentage of 17β-E_2 antioxidant activity is depicted in Fig. 4. In general, the ring B unsaturated estrogens are more potent antioxidants than 17β-E_2. Thus, 17β-Eqn, 17α-Eqn and Δ^8-E_1 are over 2.5 times more potent inhibitors of LDL oxidation than 17β-E_2, while probucol and t-resveratrol were weaker antioxidants.

The order of antioxidant activity of estrogens (Fig. 4) does not correlate with their relative binding affinities for ERα and ERβ or with their uterotropic activity: 17β-E_2 = E_1 = Eq = 17β-Eq > Δ^8-17β-E_2 > Δ^8-E_1 > Eqn > 17β-Eqn > 17α-Eq > 17α-Eqn > 17α-E_2 [36]. Thus, estrogens with higher affinity for ERs have greater uterotropic activity for example 17β-E_2 and 17β-Eq but are in general, much weaker antioxidants. In contrast, estrogens with lower uterotropic activity for example 17β-Eqn, 17α-Eqn and Δ^8-E_1, (less feminizing estrogens) are more potent antioxidants. This discordance is most likely due to the involvement of different mechanisms (genomic/non-genomic) by means of which estrogens exert their wide variety of biological effects. The data strongly suggest that the antioxidant activity of estrogens involved in the protection of LDL against oxidative modifications is expressed by mechanisms that are most likely non-genomic and do not involve ERs directly.

The dose–response data [36] further indicates that the protective effect can occur at doses that are lower than the minimum dose tested. Because all estrogen components of CEE were found to be potent antioxidants, the in vivo effects of CEE on LDL oxidation is the result of the sum of their individual activities. A pharmacokinetic study [37] further reported that after a single oral dose of CEE (2×0.625 mg), high levels of circulating total estrogens with AUCs (area under the curve) of >70 ng/h/ml were found. Thus, daily administration of CEE at regular pharmacological doses used for ERT and HRT, can give rise to levels of estrogens that are capable of protecting endogenous LDL against oxidative damage. To test this, the following in vivo study was carried out.

Three groups of postmenopausal women were selected based on whether they were on long-term CEE therapy (Group A: 0.625 mg CEE; $n = 21$), or combination CEE + progestogen therapy (Group B: 0.625 mg CEE + 5.0 mg medroxyprogesterone acetate (MPA), 10 days; $n = 20$), or not on any hormone therapy (Group C; $n = 37$). From the blood samples obtained from each of these women, LDL was isolated [36] and then subjected to copper-induced oxidation. The rate of conjugated diene formation was measured over a period of 4 h. Typical sigmoidal curves were observed and from these the lag time was measured. The mean lag times for groups A–C were 68 ± 4, 69.75 ± 6 and 50 ± 3 min, respectively (Fig. 5). The difference in lag time indicates that diene formation was significantly ($P < 0.01$) delayed (increased lag time) in both groups A and B compared to group C. No significant difference in lag time between groups A and B were observed. These data indicate that both ERT and HRT in postmenopausal women inhibits the oxidation of LDL to a similar extent. The data also indicate that long-term administration of CEE to postmenopausal women can protect endogenous LDL against

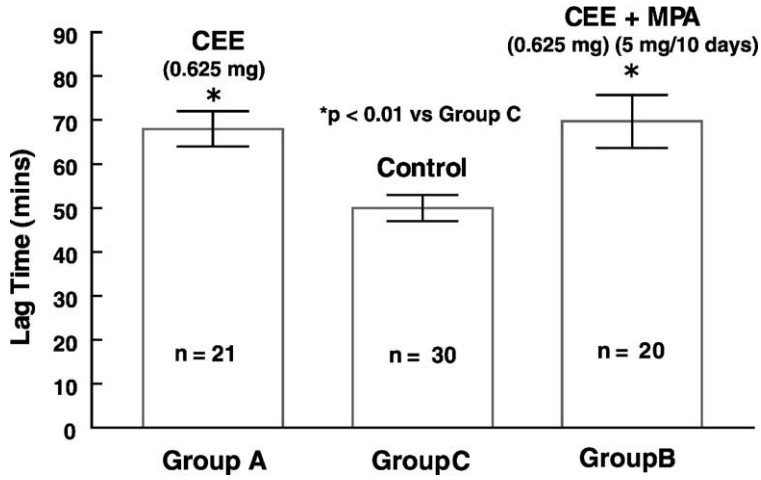

Fig. 5. Effect of CEE and CEE + MPA on the oxidation of LDL ([36] with permission).

oxidative damage and that the long-term administration of sequential MPA in conjunction with CEE does not attenuate the protective effects of estrogens as had been reported in a short-term (30 days) progestin use study [38]. Our data further suggest that beneficial effects of ERT and HRT on LDL oxidation are more likely to occur after long-term therapy. Furthermore, both in vitro and in vivo studies indicate that CEE and its individual components have potent antioxidative properties that may have long-term beneficial effects.

A number of studies [36,39–42] have indicated that antioxidants such as Vitamin E, quercetin, t-resveratrol and probucol, may protect against atherosclerosis by various mechanisms, including inhibition of LDL oxidation. If oLDL is important in the etiology of atherosclerosis, then the results of our studies indicate that estrogens in general are more effective inhibitors of LDL oxidation. Although the precise mechanism by which estrogens protect LDL against oxidation of LDL is unknown at present, it appears that these effects are not mediated via ERs and several hypotheses have been proposed [43].

In conclusion, these in vitro and in vivo studies indicate that long-term therapy of postmenopausal women with ERT/HRT was associated with the prolongation of the lag phase of LDL oxidation. Thus, some of the cardioprotective benefits thought to be associated with ERT/HRT and red wine consumption may be due to the ability of their components to protect LDL against oxidative modifications.

1.6. Potential neuroprotective effects of equine estrogens

Recent observational studies along with the discussion of data in the previous section indicate that estrogen use in postmenopausal women is associated with reduced (25–70%) risk of Alzheimer's disease [44–47]. In the following studies, we have investigated the neuroprotective effects of equine estrogens against neurotoxins such as oLDL and glutamate. These in vitro studies were carried out in a neuronal-like PC12 cell line derived from rat adrenal pheochromocytoma cells and an immortalized mouse hippocampal cell line HT22 [48,49].

The PC12 cells after plating, were cultured in the presence or absence of pharmacological concentrations (0.1–50 μM) of estrogens followed by addition of oLDL (5–12.5 μg per well). The results indicate that increasing concentrations of oLDL resulted in a dose-dependent increase in cell death in absence of estrogens [49]. With the exception of 17α-E_2 which was relatively ineffective, all of the remaining equine estrogens tested were found to be neuroprotective against oLDL in a typical dose-dependent manner. For comparative purposes, the potency of 17β-E_2 was set at 100%, and the activity of the remaining estrogens was expressed as the percentage of 17β-E_2 and the results are shown in Fig. 6. These comparisons indicate that the estrogens tested could be divided into three groups. In addition to 17α-E_2, which was relatively ineffective, the least potent estrogens were 17α-Eqn, 17α-Eq, E_1, and 17β-E_2. In contrast, 17β-Eq, Eq, 17β-Eqn, and Eqn were as much as two times more potent than the previous group. However, the most potent neuroprotective estrogens were the two Δ^8-estrogens, i.e. Δ^8-E_1 and Δ^8,17β-E_2. These two novel estrogens were nearly 10 times as potent as 17β-E_2 (Fig. 6).

Glutamate is the major excitatory neurotransmitter in the mammalian brain and at high concentrations, this amino acid is thought to play an important role in the etiology of a number of human neurological disorders such as stroke, hyperglycemia, trauma, epilepsy and chronic neurodegenerative states such as dementia, amyotrophic lateral sclerosis, Huntington's disease and Alzheimer's disease [50–53]. We have used the PC12 and HT22 cell lines to investigate glutamate-induced neuronal degeneration and its prevention

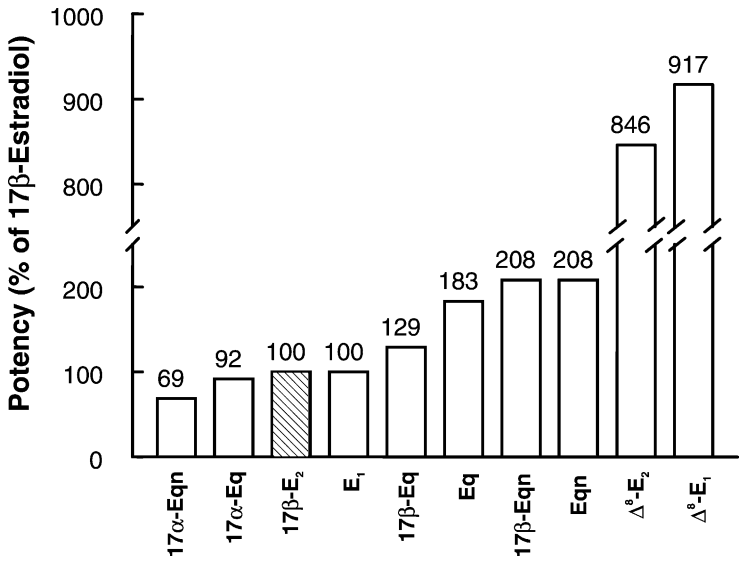

Fig. 6. Normalized neuroprotective potencies of various estrogens expressed as percentage of 17β-estradiol ([49] with permission).

by equine estrogens. These experiments were carried out by methods similar to those described for oLDL [49]. The results indicate that cell toxicity in both cell lines was directly proportional to the concentration of glutamate. The LD_{50} of glutamate was 1.8 mM for HT22 cells and 3 mM for PC12 cells. All estrogens tested were found to be neuroprotective against glutamate-induced cell death in a dose related manner. However, these estrogens differed extensively with respect to their neuroprotective potencies. In both cell lines, the Δ^8-ring B unsaturated estrogens were the most neuroprotective while the classical estrogens 17β-E_2, E_1 and 17α-E_2 were the least potent. In both cell lines, the Δ^8-estrogens were 5–25 times more potent than 17β-E_2. The order of potencies were: Δ^8,17β-$E_2 \geq \Delta^8$,E_1 > 17β-Eqn > 17α-Eqn ≥ Eqn > 17β-Eq ≥ Eq ≥ 17α-Eq > 17β-E_2 > 17α-E_2 > E_1. This order is different from that observed against oLDL: Δ^8-E_1 > Δ^8,17β-E_2 > Eqn > 17β-Eqn > Eq > 17β-Eq > E_1 = 17β-E_2 ≥ 17α-E_2 > 17α-Eq < 17α-Eqn. These data further indicate that the order of neuroprotective potency of the equine estrogens and their antioxidant potencies discussed above, do not correlate fully. The main difference between the neuroprotective activity and the antioxidant activity was that the antioxidant activity was essentially similar for 17β- and 17α-hydroxyestrogens. In contrast the 17β-hydroxyestrogens were substantially better neuroprotectors than their 17α-hydroxy stereoisomers. These data further suggest that the neuroprotective effects of equine estrogens may be important in attenuating the oxidative stress induced by reactive oxygen species (ROS), but the protection against neurotoxins such as oLDL and glutamate also involves other mechanisms, and some of these are discussed next.

1.7. Regulatory factors involved in apoptosis play a role in the neuroprotective effects of equine estrogens against glutamate-induced neuronal cell death

HT22 cells which are reported to be devoid of estrogen receptors [54,55], were used to investigate the effects of equine estrogens on glutamate-induced apoptosis by assessing DNA fragmentation, levels of anti-apoptotic protein Bcl-2, and levels of pro-apoptotic protein Bax. After exposure of HT22 cells to 18 h of glutamate (5–20 mM), DNA fragmentation was induced. Addition of 10 μM Δ^8,17β-E_2 but not 17β-E_2 totally inhibited DNA fragmentation. Glutamate (5–7 mM) alone resulted in 30–50% cell death (LDH release assay) in 8 h. Western blotting indicated that the levels of Bcl-2 decreased by 50% ($P = 0.002$), while the expression of Bax increased by 25% ($P = 0.01$) over baseline values. In presence of 5 mM glutamate, and various amounts of estrogens (0.01–10 μM) the cell death was prevented and this was associated with a significant ($P = 0.04$) increase in Bcl-2 protein levels. Bax levels decreased gradually and attained significance ($P = 0.02$) only at higher concentration (10 μM) of estrogens. In presence of higher concentration (7 mM) of glutamate which induced over 50% of cell death, only Δ^8,17β-E_2 (0.1 μM) prevented cell death, while 17β-E_2 was ineffective. These preliminary data [56] indicate that Δ^8-estrogens appear to be more effective inhibitors of apoptosis than the classical estrogens. These results further indicate that glutamate-induced cell death results in the down-regulation of anti-apoptotic protein Bcl-2 and up-regulation of pro-apoptotic protein Bax. Novel equine estrogen Δ^8,17β-E_2 was a more potent inhibitor of DNA fragmentation by up-regulation of Bcl-2 and down-regulation of Bax. Moreover, since the HT22 mouse hippocampal cell line is devoid of estrogen receptors, the anti-apoptotic effects of estrogens observed mostly likely involve non-genomic mechanisms.

1.8. Summary and conclusions

1. All equine estrogens bind with human ERα and ERβ.
2. Some of the ring B unsaturated estrogens bind with ERβ with 2–4 times greater affinity.
3. All estrogens are potent antioxidants.
4. The oxidation of LDL is inhibited differentially by various estrogens and other antioxidants.
5. The unique ring B unsaturated estrogens were the most potent, and t-resveratrol, the red wine component, was the least potent.
6. Long-term CEE or CEE + MPA administration to postmenopausal women protects LDL against oxidation.
7. Neurotoxic effects of oLDL can be inhibited differentially by various estrogens, with the Δ^8-estrogens being the most potent.
8. The neuroprotective benefits associated with estrogen therapy may be due in part to the suppression of oLDL neurotoxicity.
9. Glutamate, the excitatory neurotransmitter, is neurotoxic to neuronal cells at high concentration.
10. Glutamate induces apoptosis in neuronal cells that results in DNA fragmentation, down-regulation of anti-apoptotic protein Bcl-2, and up-regulation of pro-apoptotic protein Bax.
11. Glutamate neurotoxicity can be inhibited differentially by various equine estrogens with Δ^8-estrogens being the most potent.
12. Δ^8-Estrogens can prevent glutamate-induced apoptosis by increasing Bcl-2 protein and decreasing Bax protein levels.

Further chemical modifications of Δ^8-estrogens may provide compounds that have selective affinity for ERβ alone and these may be useful in the prevention of neurodegenerative diseases in both women and men.

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