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Equine estrogens induce apolipoprotein E and glial fibrillary acidic protein in mixed glial cultures

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Abstract

Premarin, which contains several equine estrogens, as well as estradiol (E2) as a minor component, is widely used for replacement therapy of estrogen deficits, but little is known of its direct actions on brain cells. In mixed glial cultures, apolipoprotein E (apoE) and glial fibrillary acidic protein (GFAP) are induced by estrogens. GFAP induction showed an inverted-U shape E2 dose response, with a maximum induction at 1 pM, whereas apoE mRNA induction was greatest at 100 pM. GFAP and ApoE mRNAs were induced by equine estrogens in the following order: E2 = equilin > estrone > 17α-dihydroequilenin. However, the induction of apoE secretion by 17α-dihydroequilenin was as effective as by the other estrogens. The greater response of apoE secretion than GFAP mRNA induction to 17α-dihydroequilenin might be therapeutically important because of the glial scarring during brain lesions, in which GFAP induction has a major role in inhibiting neurite outgrowth, whereas apoE secretion supports neurite outgrowth.

Keywords: Equine estrogens; Premarin; Glia; Apolipoprotein E; Glial fibrillary acidic protein; Plasticity

Little is known about the neurobiological activities of individual equine estrogens in Premarin, despite the extensive use of Premarin as a therapy for menopause since 1942 [1–3,6]. Premarin, so called because of its source, '*pre*gnant *mar*es ur*in*e', contains more than ten sulfate-conjugated estrogens (conjugated equine estrogens; see Table 1 for its main components). The activities of Premarin components on brain cells are pertinent to controversies about hormone replacement in Alzheimer disease and other age-related cognitive impairments [3].

In vitro, Premarin promotes neurite formation and protects against oxidative damage [4]. In vivo, Premarin induced neurofilament (68 kDa) mRNA in spinal ganglion neurons of adult ovariectomized rats [13]. However, little is known about the neurobiological activities of the many steroids in Premarin. Estrone, its principal estrogen and a weak estrogen also found in human plasma, is about 50% of the total steroid content, whereas 17β-estradiol (E2), the major circulating estrogen of young adult women, is present at about 10%. Premarin also contains many equine estrogens not found in human blood, as well as lesser amounts of progestins and androgens [1,6]. The best studied equine estrogen is equilin, which is second in abundance (25%) to estrone (Table 1). Equilin also promoted outgrowth of neurites in vitro through *N*-methyl-D-aspartate receptors [3]. In vivo 17α-dihydroequilenin (10% of Premarin) was as effective as E2 in increasing dendritic spine density in hippocampal CA1 neurons of ovariectomized rats [20]. However, in promoting uterine growth, 17α-dihydroequilenin is less active than E2 [2,8] (see Table 1), which could indicate a tissue difference in estrogen receptors (ER): the uterus has primarily ERα, whereas CA1 neurons have both ERα and ERβ [10].

This study examined astrocytic responses to equine estrogens. In vivo, the E2-dependent sprouting of hippocampal neurons is mediated by apolipoprotein E (apoE) [9,16–18]. ApoE is secreted by astrocytes and is a carrier of lipoproteins

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Table 1
Estrogenic components of Premarin: regulation of GFAP and apoE in mixed glia and comparison with activities on rat uterus

Estrogen (% in Premarin)[a]	Percentage of vehicle control								Uterine growth[b] (%)	K_a (nmol) uterine cytosol[c]
	GFAP mRNA (Northern blot)		GFAP protein (ELISA)		ApoE mRNA (Northern blot)		apoE secreted (Western blot)			
	Dose (pM)		Dose (pM)		Dose (pM)		Dose (pM)			
Premarin (10 ng/ml)[d]		101 ± 3		90 ± 12		134 ± 36		186 ± 43[e]	–	–
17β-Estradiol (4.5–12%)	1	258 ± 9[e]	1	158 ± 7[e]	1	160 ± 7[e]	1	110 ± 9	305	13
	100	110 ± 4	100	102 ± 4	100	220 ± 10[e]	100	177 ± 10[e]		
Estrone (52.5–60%)	1	113 ± 1	1	126 ± 9	1	96 ± 5	1	150 ± 14[e]	350	3.4
	100	216 ± 2[e]	100	117 ± 3	100	205 ± 9[e]	100	101 ± 4		
Equilin (22.5–30%)	1	157 ± 9[e]	1	118 ± 11	1	212 ± 24[e]	1	187 ± 11[e]	408	0.3
	100	290 ± 3[e]	100	99 ± 5	100	187 ± 11[e]	100	162 ± 3[e]		
17α-Dihydroequilenin (4.5–12.5%)	1	128 ± 8		–	1	101 ± 2	1	140 ± 8[e]	120	0.06
	100	77 ± 9			100	115 ± 4	100	186 ± 21[e]		

[a] The components in Premarin, with a range of variations between preparations [2].
[b] Data from Ref. [2]. Bioassay of total uterine growth (wet weight); female rats aged 21 days were given s.c. injections of 2.0 g of the named estrogen for 3 days; uterine wet weight was measured on the fourth day and expressed as a percentage of vehicle control. Dorfman et al. [7] also found greater uterotrophic activity of equilin than estrone.
[c] Data from Refs. [2,9]. Binding of E2 and equilin to uterine cytosol from 21 day female rats with E2 showed a single class of binding sites (Scatchard analysis); K_a values were determined by competition of unlabeled steroid against bound [^{3}H]E2.
[d] Premarin (Wyeth–Ayerst, Philadelphia, PA); vehicle control for Premarin (10 ng/ml; Wyest–Ayerst) consisted of the non-steroidal components; this vehicle did not show any activities. The absence of Premarin's effect on GFAP, but its induction of apoE is consistent with the concentration of E2 in 10 ng/ml of Premarin
[e] Data (mean ± SEM) of three independent experiments are expressed as a percentage of vehicle; $*P < 0.05$ (analysis of variance (ANOVA)).

to outgrowing neurites [9]. Because apoE mRNA and secretion are induced by E2 [15], we characterized responses to equine estrogens. Responses of apoE were compared with glial fibrillary acidic protein (GFAP), an astrocyte cytoskeletal component with a maximum transcriptional response at 1 pM, which is mediated through a functional estrogen response element in the upstream promoter [17]. Mixed glial cultures were used because in monotypic astrocyte cultures, apoE expression by astrocytes is not sensitive to E2, whereas apoE mRNA and apoE secretion are robustly induced by E2 when astrocytes are co-cultured with microglia [15]. Mixed glial cultures from neonatal rat cortex were grown to confluence [15]. Steroid (Steraloids, Inc., Newport, RI) was freshly prepared in 80% ethanol and diluted to 0.008% ethanol; ethanol vehicle was added to controls. Media were assayed for apoE protein by Western blot, GFAP protein by enzyme-linked immunosorbent assay (ELISA), and cell RNA was assayed by Northern blot [15,17].

We first examined the E2 dose response of apoE mRNA induction with reference to GFAP mRNA in the same RNA samples. The induction of apoE mRNA by E2 was less sensitive than GFAP, with an EC_{50} that was about ten-fold higher (Fig. 1), and which differed from the sharp dose-dependent inverted-U shape of GFAP mRNA induction. The GFAP dose response in these mixed glia is identical to that in monotypic astrocyte cultures [18]. Thus, the regulation of GFAP by E2 differs from ApoE in two respects: it is more sensitive to E2 and does not require the presence of microglia [15].

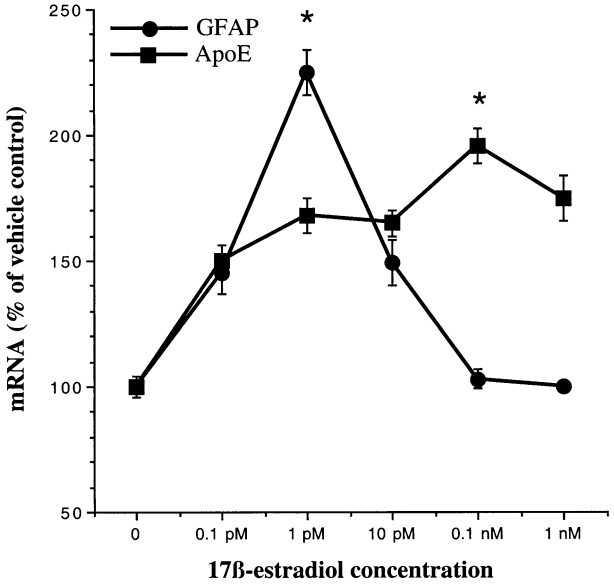

Fig. 1. ApoE and GFAP mRNAs induction in mixed glia by E2 at 24 h. ApoE mRNA has a different dose response than GFAP mRNA with a progressive increase across E2 doses examined. GFAP mRNA shows a dose-dependent inverted-U shape response. Data (Northern blot; mean ± SEM) of three independent experiments expressed as a percentage of vehicle control; $*P < 0.05$, ANOVA.

Premarin (Wyeth–Ayerst, Philadelphia, PA) was used at 10 ng/ml, the dose that showed maximum protection against H_2O_2- and β-amyloid peptide-induced neurotoxicity [4]. GFAP mRNA and protein were not affected by 10 ng/ml of Premarin, while apoE secretion was induced. There was a trend in apoE mRNA induction (see Table 1). Vehicle control (also Wyeth–Ayerst) consisting of non-steroidal components of Premarin did not alter GFAP or apoE.

As a first approach to defining the response of apoE and GFAP to equine estrogens, we chose the doses of 1 and 100 pM, which are the respective maximum E2 responses of apoE mRNA (100 pM) and GFAP mRNA (1 pM) (see Fig. 1). ApoE mRNA induction by equine estrogens followed the rank order of response at the same dose: equilin = E2 > estrone > 17α-dihydroequilenin (Table 1). However, equilin showed similar induction of apoE mRNA at both 1 and 100 pM, yet greater induction of GFAP mRNA at 100 pM than 1 pM. The responses to equilin were greater than to 17α-dihydroequilenin in the induction of GFAP and apoE mRNAs, parallel to the greater uterotrophic effect of equilin, as noted above. Secreted apoE was increased by all estrogens, with greater sensitivity than for induction. More detailed dose responses are needed to resolve possible differences between E2, equilin, and 17α-dihydroequilenin.

These responses differ considerably from the relative rank in binding to uterine cytosolic ERs, e.g. both equilin and 17α-dihydroequilenin have greater than ten-fold lower K_a than estrone or E2 [2,8] (summarized in Table 1). These differences could reflect interactions with different ER subtypes. Uterine cytosols contain mainly ERα; in most astrocytes ERβ is predominant [11], although ERα is also detected [11]; microglia have both receptors [19]. Moreover, equine estrogens could also differ as cell-type selective estrogen receptor modulators (SERMs). Variations between batches of Premarin in its components (Table 1) could have unexpected effects on these interacting systems.

These findings extend the direct effects of equine estrogens in Premarin on neuronal survival and sprouting [3,4,20] to apoE, an astrocyte secretion, with many activities on brain cell functions. One phase of the E2-supported neurite sprouting is dependent on apoE [16–18]. Due to the importance of GFAP to glial scarring in vivo, it might be possible to optimize hormone replacements with 17α-dihydroequilenin to enhance neurite outgrowth through apoE secretion, without stimulating GFAP. The complexity of GFAP control by sex steroids is well documented by us and others (reviewed in Refs. [12,17]). In particular, the direction of GFAP responses to sex steroids changes from induction to repression ('transcriptional inversion') depending upon brain region in vivo, and presence or absence of neurons or its regional specificity in glial cultures in vitro.

The responses of apoE to estrone are also of interest because this estrogen is also produced by adipose cells [14]. Increased circulating estrone is associated with obesity

in postmenopausal women and is a protective factor in osteoporosis, as well as a risk factor in endometrial cancer. Moreover, estrone, by inducing apoE as shown here, could support apoE-dependant aspects of synaptic remodeling, which raises the possibility that body fat content in both sexes could indirectly influence neuronal plasticity through increased circulating estrone.

Lastly, these in vitro models could be used to study the metabolism of constituents of Premarin. In vitro, Premarin is neuroprotective against hydrogen peroxide or the amyloid β-peptide-induced toxicity [4]. However, 4-hydroxyequilenin, a catechol estrogen produced from equilin by P450 isozymes, readily autooxidizes to *o*-quinones which are directly DNA damaging [5]. These processes could be enhanced by the increased oxidative stress in the brain during aging and Alzheimer disease. As primary glia from aging brains retain in vivo phenotypes of activation in vitro [11], models with glia derived from adult brains of different ages may be used to optimize hormone replacement therapy for different age groups.

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