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PRELIMINARY REPORT

Superior and Distinct Antioxidant Effects of Selected Estrogen Metabolites on Lipid Peroxidation

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The effect of the estrogen metabolites, 4-hydroxyestrone and 17α-dihydroequilin (metabolites of estradiol-17β and equilin, respectively), were examined for antioxidant effects on plasma and lipoprotein lipid peroxidation. Lipid peroxidation was evaluated by products of both fatty acid (thiobarbituric acid–reactive substances [TBARS]) and cholesterol (oxysterols) oxidation from lipoproteins or whole plasma. Although all estrogens significantly reduced lipid peroxidation, 4-hydroxyestrone was far more potent than either equilin or 17α-dihydroequilin in inhibiting TBARS formation in lipoproteins induced by Cu^{2+}. Similar effects were also noted on TBARS formation in THP-I macrophages in culture. However, 17α-dihydroequilin (along with equilin) strongly inhibited oxysterol formation, whereas 4-hydroxyestrone was ineffective. These studies suggest that different estrogens might act preferentially on distinct lipid substrates in exhibiting antioxidant effects.
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THE CARDIOPROTECTIVE effect of estrogens in postmenopausal women has been well documented.[1] Considerable attention has focused on the positive changes noted in high-density lipoprotein levels,[2,3] but the mechanism of the beneficial effect of estrogen is not entirely clear. Recent studies have suggested that lipoprotein changes may account for only 25% to 50% of the cardioprotective effect of estrogens, indicating the possibility of other mechanisms.[2] Recent studies also demonstrated that estrogen can inhibit low-density lipoprotein (LDL) peroxidation both in vitro[4,5] and in vivo.[6] Indeed, it was demonstrated that the antioxidant properties were related to the estrogen structure, with equine estrogens (eg, equilin) being more potent in this respect.[5] Since the biological effects of estrogens are believed to be mediated to a considerable degree through their metabolites,[7] we examined the antioxidant properties of estrogen metabolites. Attention was focused on 4-hydroxyestrone, a reactive metabolite of estradiol-17β,[8] and 17-dihydroequilin, a metabolite of the major equine estrogen, equilin.[9] The data are compared with those obtained with other estrogens, including 17α-dihydroequilenin, an equine estrogen known to possess plasma cholesterol-lowering properties.[10] In this communication, we show that the antioxidant potential of estrogen metabolites varies depending on the lipid substrate (fatty acid or cholesterol). Although differences in the antioxidant potential of various tocopherols and carotenes have been recognized,[11,12] little attention has been paid to their discriminatory effects on cholesterol and fatty acid peroxidation in lipoproteins.[5,13]

MATERIALS AND METHODS

Estrogens were purchased from either Sigma Chemical (St Louis, MO; estradiol-17β, equilin, and equilenin) or Steraloids (Wilton, NH; 4-hydroxyestrone, 16-hydroxyestrone, 17α- and 17β-dihydroequilin, and 17α-dihydroequilenin).

Isolation of Plasma and Lipoproteins

Plasma was obtained by centrifugation of blood samples collected from fasting donors at 1,500 × *g* for 15 minutes at 4°C. None

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of the donor subjects were on antioxidant or estrogen therapy. Plasma lipoproteins were sequentially isolated by ultracentrifugation, dialyzed, and stored at 4°C after purging with nitrogen as described previously.[5] Briefly, very-low-density lipoproteins (VLDL) and LDL were isolated with solid KBr at a density of less than 1.006 (42,000 rpm for 18 hours) and a density of 1.006 to 1.063 (42,000 rpm for 20 hours), respectively, using a Beckman L3-50 ultracentrifuge and Ti 50.3 rotor (Beckman Instruments, Fullerton, CA). The lipoprotein solution (2 to 3 mL) was flushed with N_2 and dialyzed Dulbecco's phosphate-buffered saline ([PBS] 0.037 mol/L $Na_2HPO_4 \cdot 7H_2O$, 0.0018 mol/L K_2HPO_4, 0.046 mol/L NaCl, and 0.003 mol/L KCl, pH 7.4) without EDTA for 24 hours with two changes of dialyzing solution (4 L). EDTA-free solutions were used for dialysis in order to eliminate its biphasic influence on lipoprotein oxidation,[14] especially under conditions of low-dose estrogen. Under these conditions, basal levels (without Cu^{2+}) of peroxidation in lipoproteins were negligible (optical density, 0.001). Under our conditions, this corresponds to 0.2 to 0.3 nmol/L thiobarbituric acid–reactive substances [TBARS]) formed, as opposed to 20 to 40 nmol/L in samples with Cu^{2+}.

Determination of the Estrogen Effect on Lipoprotein Peroxidation (using TBARS) and Plasma Oxysterol Formation

The effect of estrogens on lipoprotein peroxidation was determined in two ways. First, 100 to 200 μL VLDL (130 to 545 μg protein) or 50 to 100 μL LDL (125 to 565 μg protein) in PBS were incubated with cupric sulfate (50 mmol/L) or cupric acetate (10 mmol/L) with or without estrogen, and TBARS formation was determined by the thiobarbituric acid method of Satoh.[15] Second, cholesterol oxidation was determined by quantitation of the oxysterols formed as described by Bhadra et al.[16] In this procedure, 0.5 mL plasma was incubated with cupric ions for 16 hours, and cholesterol oxides were extracted with chloroform:methanol (2:1 vol/vol). Oxysterols were identified and quantified on the basis of the relative retention time[16] and peak area of 5α-cholestane (internal standard) by gas-liquid chromatography. The amount of estrogen used was pharmacological (7×10^{-5} mol/L) and much higher than total levels (0.2 to 1×10^{-6} mol/L) noted after estrogen therapy.[17] The concentration of estrogen metabolites in plasma after therapy has not been established.

Cell Culture and Lipoprotein Oxidation

Cultures of the THP-1 cell line (human monocyte-derived cell line obtained from The American Type Culture Collection, Rockville, MD), which actively oxidizes lipoproteins,[5] were seeded in 12-well tissue culture dishes in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mmol/L HEPES, 5×10^{-5} mol/L β-mercaptoethanol, and 2×10^{-7} mol/L phorbol myristate acetate (PMA). After 24 hours, cells were washed twice with PBS and the medium was replaced with minimal essential medium (without phenol red), 2% fetal calf serum, PMA (2×10^{-7} mol/L), VLDL (130 μg protein/mL medium), and specific estrogen (3.75×10^{-5} mol/L) and incubated for 24 hours. The next day, the cells were collected and homogenized, and TBARS formation was determined as previously described.[5]

Statistical Analysis

Statview 512+ (version 1.2; Abacus Concepts, Berkeley, CA) was used for statistical analysis of the data. One-way ANOVAs were performed for each dependent variable to determine whether there were differences among groups. Multiple comparisons among means were made using the Scheffé F test.

RESULTS

Table 1 shows the effect of various estrogen metabolites on TBARS formation from VLDL and LDL under the influence of Cu^{2+} ions. Equilin was included for comparative purposes, since it was shown to be potent among parent estrogens, being more effective than estradiol-17β.[5] As expected, all estrogens inhibited TBARS formation in Cu^{2+}-induced LDL and VLDL peroxidation as compared with the control ($P < .01$). However, 4-hydroxyestrone appears to be more effective than other estrogens in inhibiting LDL and VLDL peroxidation mediated by cupric ions ($P < .01$). It should be noted that 4-hydroxyestrone was far superior to 17α-dihydroequilin ($P < .01$) in antioxidant effects. Further experiments were focused on the comparative effects of two potent estrogen metabolites (4-hydroxyestrone and 17α-dihydroequilin).

To compare the relationship of estrogen concentration to the decrease in TBARS formation, we determined the effect of various dosages of estrogens on VLDL peroxidation at constant Cu^{2+} concentration (Fig 1). The antioxidant effect of 4-hydroxyestrone was far greater than that of either equilin or 17α-dihydroequilin at all concentrations.

The effect of estrogen metabolites on TBARS formation in human monocyte-derived macrophages was consistent with results obtained using lipoprotein incubations, with 4-hydroxyestrone being most potent (Table 2). Equilin and its metabolite (17α-dihydroequilin) significantly ($P < .05$) suppressed fatty acid peroxidation in cells, and no difference was evident between these two estrogens.

The antioxidant potential of 4-hydroxyestrone and 17α-dihydroequilin on peroxidation of the cholesterol moiety of lipoprotein was compared in whole plasma. Table 3 shows that 17α-dihydroequilin strongly inhibited the formation of oxysterols by cupric ions ($P < .01$), and 4-hydroxyestrone

Table 1. Effect of Specific Estrogen Metabolites on VLDL and LDL Peroxidation (mean ± SD)

Group	TBARS Formation (nmol)	
	VLDL	LDL
Control	31.75 ± 1.39	49.77 ± 0.95
Equilin	14.21 ± 0.95*	15.98 ± 0.23*
17α-Dihydroequilin	11.31 ± 0.60*	14.50 ± 0.61*
17β-Dihydroequilin	13.23 ± 1.21*	17.56 ± 0.53*
17β-Dihydroequilenin	14.68 ± 2.88*	18.12 ± 0.77*
4-Hydroxyestrone	5.04 ± 0.20*†	5.44 ± 0.98*
16-Hydroxyestrone	25.22 ± 0.95*	26.77 ± 0.39*

NOTE. VLDL (130 μg protein) and LDL (125 μg protein) were incubated with cupric sulfate 50 μL (50 mmol/L) and estrogens (7.5×10^{-5} mol/L) for 1 hour at 37°C. Each data point is the mean of 3 determinations, except for controls (n = 4).

*Significantly different from control ($P < .01$).

†Significantly different from other estrogens ($P < .01$).

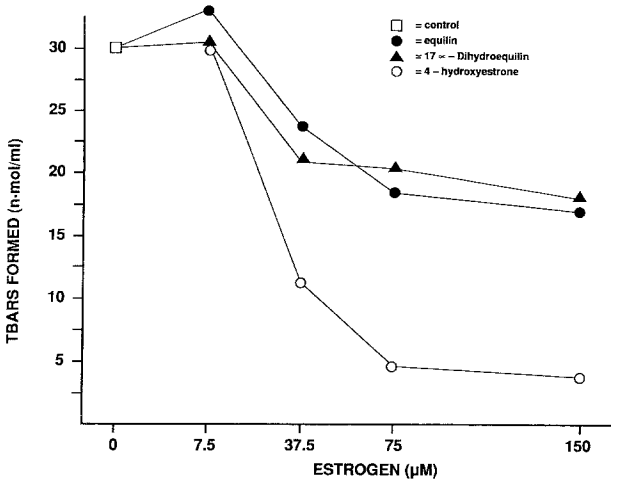

Fig 1. Effect of estrogen concentration on TBARS formation from VLDL. Each point represents the mean of 3 determinations.

had no effect. Furthermore, under the conditions used, equilin, estradiol-17β, and 17α-dihydroequilin were equally effective and reduced oxysterol formation to extremely small quantities.

In all the above experiments, equilin (equine estrogen) was used as a standard for comparative purposes. Additional experiments compared estradiol-17β (the major human estrogen) with estrone, 4-hydroxyestrone, and equilin on TBARS formation. These data are shown in Table 4. Although there were slight differences in the degree of inhibition by some estrogens as compared with Table 1, the order of effectiveness remained the same (ie, 4-hydroxyestrone > equilin, followed by estradiol-17β and estrone, respectively).

DISCUSSION

Estrogens have been shown to inhibit LDL oxidation induced by cupric ions, macrophages, or endothelial cells in vitro,[4,5] and it has been postulated that this effect is involved in protection against cardiovascular disease. Previous studies[5] noted that equine estrogens (especially equilin) were more effective in inhibiting both fatty acid and cholesterol oxidation when compared with estradiol-17β. Whether this difference in antioxidant potential exists among estrogen metabolites is not known. Furthermore, in evaluating the antioxidant potential of vitamins[11-13] on peroxidation, the focus was predominantly on fatty acids and it was assumed that the antioxidant properties would be similar on fatty acid and cholesterol oxidation.[5] In the present studies, we found that 4-hydroxyestrone (a metabolite of estradiol-17β) was significantly superior to 17α-dihydroequilin and other estrogens in inhibiting TBARS formation from LDL or VLDL or in cells. In contrast, 17α-dihydroequilin (a metabolite of dihydroequilin) was far superior to 4-hydroxyestrone in inhibiting oxysterol formation (Table 4). These findings suggest that different estrogens might preferentially act on distinct lipid substrates in inhibiting peroxidation, and that the selected effect of estrogen on substrates should be considered in evaluating the cardioprotective effect of specific estrogen preparations in clinical therapy.

Although a number of epidemiological and clinical studies are performed on the effects of estrogen therapy, plasma levels of estrogen metabolites have not been measured. We believe this information is critical for developing estrogen derivatives with greater target specificity.

Table 2. Effect of Estrogen Metabolites on TBARS Formation in THP-1 Macrophages (mean ± SD)

Estrogen	TBARS (nmol/mg protein)
Control	12.05 ± 1.72
Equilin	8.02 ± 0.53*
17α-Dihydroequilin	8.43 ± 0.99*
4-Hydroxyestrone	4.37 ± 1.14*†

NOTE. THP-1 macrophages were incubated with VLDL (130 μg) and estrogen (3.75×10^{-5} mol/L) for 24 hours, and cellular TBARS levels were measured. Each point represents the mean of 3 determinations.

*Significantly different from control ($P < .05$).

†Significantly different from other estrogens ($P < .05$).

Table 3. Effect of Estrogen Metabolites on Formation of Plasma Oxysterols (mean ± SD)

Group	Oxysterols (μg/mL)
Control	16.75 ± 2.12
Equilin	1.36 ± 0.44*†
17α-Dihydroequilin	1.02 ± 0.92*†
4-Hydroxyestrone	13.51 ± 3.70

NOTE. The plasma (500 μL) was incubated with cupric sulfate 100 μL (50 mmol/L) and 5α-cholestane (internal standard), as well as estrogens (7.5×10^{-5} mol/L), for 16 hours at 37°C, and oxysterols were quantified by gas-liquid chromatography following extraction.[16] Each point is the mean of 3 determinations.

*Significantly different from control ($P < .01$).

†Significantly different from 4-hydroxyestrone ($P < .01$).

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Table 4. Effect of Selected Estrogens on VLDL and LDL Peroxidation (mean ± SD)

Group	TBARS Formation (nmol)	
	VLDL	LDL
Control	43.84 ± 0.89	50.04 ± 1.40
Estrone	15.29 ± 0.24*†	49.88 ± 0.65†
Estradiol	11.37 ± 0.14*†	29.22 ± 0.51*†
Equilin	8.78 ± 0.59*†	11.93 ± 0.51*†
4-Hydroxyestrone	4.86 ± 0.72*†	2.55 ± 0.14*†

NOTE. VLDL (545 μg protein) and LDL (565 μg protein) were incubated with cupric acetate 200 μL (10 mmol/L) and estrogens (7.5×10^{-5} mol/L) for 2 hours at 37°C. Each data point is the mean of 3 determinations, except for controls (n = 4).

*Significantly different from control ($P < .01$).

†Significantly different from other estrogens ($P < .05$).

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