An Investigation of the Relationship Between Estrogen, Estrogen Metabolites and Blood Cholesterol Levels in Ovariectomized Rats

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ABSTRACT
17β-Estradiol (E2) has long been known for protecting against coronary heart disease by lowering cholesterol levels in premenopausal women. A recent study in our laboratory suggested that two hydroxylated metabolites of E2 possess similar hypocholesterolemic effects in male rats. This effect has been further investigated with additional estrogen metabolites in ovariectomized rats with a view toward mimicking the true postmenopausal situation in humans. Their effects in reproductive tissues were also evaluated histologically. Fundamentally, the following issues were addressed: (1) Do oxidized metabolites of estradiol lower total cholesterol levels? (2) Can a hypocholesterolemic effect be achieved without eliciting estrogenic activities on reproductive tissues? The results of this investigation showed that a number of oxygenated metabolites of estradiol can lower cholesterol levels. Among them, 4-hydroxyestradiol (4-OHE2) produced a striking hypocholesterolemic effect and a substantial uterotropic effect. 2-Hydroxyestradiol (2-OHE2), 2-methoxyestradiol (2-meoE2) and 2-methoxyestrone (2-meoE1) produced a significant decrease in cholesterol levels at doses that did not produce significant uterotropic effects.

Estrogen has long been touted as a beneficial factor in preventing cardiovascular diseases by keeping plasma cholesterol levels low in premenopausal women. Postmenopausal women lose this protection because of dramatic decreases in estrogen levels as a result of natural atrophy of the ovaries (Robinson et al., 1959; Rosenberg et al., 1981). Estrogen replacement therapy in postmenopausal women restores this protective effect against CAD (Grady et al., 1992). However, an increase in side effects, such as breast cancer, resumption of menses and weight gain, has consistently accompanied this treatment (Judd et al., 1983; Henderson et al., 1993).

Estradiol exerts a favorable cardiovascular profile through its effects on serum lipoprotein concentrations. Studies have shown that estrogen monotherapy can decrease LDL-cholesterol (Campos et al., 1990; Wallace et al., 1979). It has been shown that the induction of hepatic LDL receptor activity is the major mechanism responsible for the hypocholesterolemic effect of estrogen (Kovanen et al., 1978; Cooper et al., 1987; Srivastava et al., 1993).

Many oxygenated-estrogen metabolites have the ability to bind to ERs, and their binding affinities correlate with their biological activities quite strongly (Martucci and Fishman, 1977). Generally, a steroid will have a biological effect only on tissues that possess receptors for that steroid (Martucci and Fishman, 1993). All natural and synthetic estrogens interact with ERs.

The regional oxidative metabolism of estradiol has a profound impact on the nature of the biological response to the hormone. C16 hydroxylation leads to the formation of 16α-hydroxyestrone and estriol, both of which are fully active estrogens as measured by uterotrophicism (Fishman and Martucci, 1980). C2 hydroxylation elicits the formation of 2-hydroxyestrone and 2-meoE1, neither of which have virtually any uterotropic activity (Martucci and Fishman, 1979). We recently found that 2-OHE2 and 4-OHE2 are effective hypocholesterolemic agents in male rats and that 4-OHE2 is the more potent (Wright A and Bachmann KA, unpublished observations). The generation of hydroxy groups at specific sites on estrogens are mediated by specific cytochrome P450 enzymes (Martucci and Fishman, 1993; Suchar et al., 1996). The formation of 2-meoE1 and 2-meoE2 from 2-OHE1 and 2-OHE2 is catalyzed by COMT (Martucci and Fishman, 1993; Bolt, 1981).

Based on the aforementioned observations, we were interested in the following issues: (1) Do oxidized metabolites of estradiol play a role in estradiol’s lowering of total cholesterol levels? (2) Can a hypocholesterolemic effect be achieved with-

ABBREVIATIONS: E2, 17β-estradiol; ER, estrogen receptor; 2-OHE2, 2-hydroxyestradiol; 4-OHE2, 4-hydroxyestradiol; 2-meoE1, 2-methoxyestrone; 2-meoE2, 2-methoxyestradiol; TAM, tamoxifen; 4-OHT, 4-hydroxytamoxifen; LDL, low density lipoprotein; TCL, total cholesterol level; CYP, cytochrome P450; CAD, coronary artery (or heart) disease; OVX, ovariectomized; AEBS, antiestrogen binding sites.
out eliciting estrogenic activities on reproductive tissues? We investigated these issues in ovariectomized rats with several oxygenated estradiol metabolites.

**Experimental Procedures**

**Materials.** E2, 4-OHE2, 2-OHE2, 2-meoE2, 2-meoE1, 4-OHT and TAM were purchased from Sigma Chemical (St. Louis, MO). Their chemical structures are shown in figure 1. The reagent kits that were used to measure serum TCL levels were also purchased from Sigma Chemical.

**Animals.** Female, virgin Sprague-Dawley rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN). They were 200 to 225 g, 10- to 11-week-old rats and were used in groups of four or five. All rats were kept in the vivarium at 24°C on a 12-hr light/dark cycle. The animals had free access to standard chow (#8604, obtained from Harlan Teklad) and water. Food was withheld for 8 hr before death.

**Surgical procedures.** Rats (except for intact controls) were anesthetized using ketamine hydrochloride (100–120 mg/kg) and xylazine hydrochloride (24 mg/kg) intramuscularly. A small midline dorsal skin incision (1–2 cm) was made just caudal to the 13th ribs. Bilateral ovariectomy was performed according to the procedures described by Waynforth and Flecknell (1992). Animals were kept warm during the procedure and recovery. On recovery from anesthesia, animals were randomly sorted into experimental groups of four or five rats each.

**Treatment.** There were two separate experiments. In each experiment, there were both intact control and ovariectomized control groups as shown in tables 1 and 2. In all cases, all compounds were administered by gastric gavage, and 1% methylcellulose was used as the vehicle. The total volume of solution gavaged for each dose was controlled to be 1 to 1.5 ml.

In the first experiment (experiment 1), 4-OHE2 was administered in doses of 1 and 1.5 mg/kg with a view toward identifying a maximum effective dose. E2 was administered for comparison in a dose of 1 mg/kg. TAM (1 mg/kg) and 4-OHT (0.8 mg/kg) were administered with a view toward identifying which antiestrogen is more effective in blocking the hypercholesterolemic and uterotrophic effects of estradiol and its metabolite.

In a second experiment (experiment 2), E2, 2-OHE2, 2-meoE1, 2-meoE2 and 4-OHE2 were each administered in doses of 0.5 mg/kg to compare their biological activities. 4-OHE2 was also given at a dose of 0.05 mg/kg to mimic physiological conditions. 4-OHT (1.5 mg/kg) was used to determine whether the effects of estradiol and oxidized estrogen metabolites on cholesterol and on reproductive tissues were mediated through ERs.

The treatment and vehicle groups were given one daily dose for 7 consecutive days, and each dose followed the previous one by 24 hours. In experiment 1, the interval between surgery and the beginning of treatment was 14 days. Subsequently, in experiment 2, the interval was 7 days, since we learned that a 7-day interval was long enough for uteri to atrophy.

**Serum cholesterol measurement.** Blood samples were collected into sterile silicone-coated tubes (Terumo Venoject Evacuated Specimen Tubes) by cardiac puncture 24 hr after the last treatment. Animals were placed under carbon dioxide anesthesia for 1 to 2 min before we obtained 3 ml of whole blood. After collection, animals were killed using CO2 anesthesia and cervical dislocation. Serum was then separated with a bench top centrifuge running at 3000 rpm for 15 min at room temperature. After separating the serum, the samples were immediately analyzed to determine concentrations of TCL. TCL levels were measured colorimetrically with reagents obtained from Sigma Chemical (Kit 352). Measurements were made using a Beckman DU640 spectrophotometer (Beckman, Fullerton, CA). The coefficient of variation for total cholesterol measurement is 1.4%.

**Histological study of uteri.** After death, uteri were removed, weighed and fixed in 10% neutral buffered formalin. Preserved uteri were stored for 1 month before histological study. Formalin-fixed uteri were processed for conventional paraffin embedding with hematoxylin and eosin staining. The sections were studied in our laboratory using a light microscope (Leica, Galen III) fitted with a micrometer. Epithelial cell height, myometrial thickness and endometrial stromal thickness were measured perpendicularly to the long side of the luminal oval at ×80 with the micrometer, and endometrial stromal eosinophil infiltrates were evaluated by counting the number of eosinophils within each field. The measured raw data were then converted to a scale of 1 to 7. For instance, each uterine wet weight value was divided by 100 mg, and each measured value for uterine stromal expansion, uterine epithelial height and uterine myometrial thickness was multiplied by 10/mm to give final numbers for each parameter ranging from 1 to 7, which were then summed to give the total estrogenicity score. The purpose for this conversion was to confer equal weights to each measured parameter for the overall estrogenicity score.

**Statistics.** One-way ANOVA, a Student-Newman-Keuls test and the Mann-Whitney test were used to analyze for differences among experimental groups for each parameter (TCL, uterine weight, uterus/body mass ratio and total estrogenicity score). Differences were considered significant at P < .05.

**Results**

**Body weight.** Ovariectomy was associated with a 5.4% weight gain compared with a 3.4% weight loss among intact controls during the 21-day period after surgery and before death in experiment 1, table 1). In experiment 2, OVX rats exhibited a 3.8% loss in weight compared with an 8.5% loss for intact controls during the 14-day period after surgery. The differences in weight losses (OVX vs. intact controls) were not significantly different in either experiment.

**Uterus.** The effects of each treatment in experiment 1 and 2 are presented in tables 1 and 2 respectively. As expected, uterine wet weights of the OVX rats are 40% to 48% lower than for the intact controls in both experiments (P < .01). E2 at doses of 0.5 and 1 mg/kg completely restored uterine

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Fig. 1. Structures of 2-OHE2, 4-OHE2, 2-meoE1, 2-meoE2, TAM and 4-OHT.
weights to the intact control levels after a 7-day treatment, and produced significantly heavier uteri than those observed in the OVX control group rats (P < .01).

4-OHE$_2$ at doses of 0.5 mg/kg and 1 mg/kg also restored the uterine wet weight to the intact control level and significantly increased weights compared with the OVX control rats (P < .01; tables 1 and 2). 4-OHE$_2$ at 1.5 mg/kg did not alter uterine weight any more than the 1.0 mg/kg dosage. At a low dose of 0.05 mg/kg, 4-OHE$_2$ increased the uterine weight relative to OVX controls, but the increase failed to achieve statistical significance. However, the ratio of uterine/body mass was significantly increased even at this low dose. On the other hand, 2-OHE$_2$, 2-meoE$_1$ and 2-meoE$_2$ at 0.5 mg/kg showed no uterotrophic effect (tables 2 and 3).

In experiment 1, E$_2$ (1 mg/kg) with TAM was less effective than E$_2$ alone in restoring uterine weight; however, uterine weight was still significantly greater than for OVX controls, but the increase failed to achieve statistical significance (figs. 2 and 3). TAM appeared to be ineffective in blocking the hypothalamic effects of E$_2$ though the effect was not statistically significant (fig. 2). TAM seemed to be ineffective in blocking the hypocholesterolemic effect of E$_2$. 4-OHT may have partially blocked the hypocholesterolemic effects of E$_2$ though the effect was not statistically significant (fig. 2). In experiment 2, E$_2$ at a dose of 0.5 mg/kg produced a 60.6% decrease in total cholesterol levels. At the same dose, 4-OHE$_2$ virtually depleted total cholesterol levels (fig. 3). 4-OHE$_2$ at 1.5 mg/kg produced a 98.4% decrease in total cholesterol levels (fig. 3). TAM appeared to be ineffective in blocking the hypocholesterolemic effect of E$_2$. 4-OHT may have partially blocked the hypocholesterolemic effects of E$_2$ though the effect was not statistically significant (fig. 2). In experiment 2, E$_2$ at a dose of 0.5 mg/kg produced a 60.6% decrease in total cholesterol levels. At the same dose, 4-OHE$_2$ virtually depleted total cholesterol levels (fig. 3). 4-OHE$_2$ at 1.5 mg/kg produced a 98.4% decrease in total cholesterol levels (fig. 3). TAM appeared to be ineffective in blocking the hypocholesterolemic effect of E$_2$. 4-OHT may have partially blocked the hypocholesterolemic effects of E$_2$ though the effect was not statistically significant (fig. 2). In experiment 2, E$_2$ at a dose of 0.5 mg/kg produced a 60.6% decrease in total cholesterol levels. At the same dose, 4-OHE$_2$ virtually depleted total cholesterol levels (fig. 3). 4-OHE$_2$ at 1.5 mg/kg produced a 98.4% decrease in total cholesterol levels (fig. 3).

### Histological results

Uterine sections obtained from the intact controls, E$_2$-treated and 4-OHE$_2$-treated rats show epithelial lining cells with typical elongated cell bodies and elongated, diffuse nuclei (fig. 4). Epithelial cells from OVX control rats, 2-OHE$_2$, 2-meoE$_1$, and 2-meoE$_2$-treated rats were more cuboidal in appearance with smaller, darker staining nuclei (fig. 4). Uterine epithelial cell height was 54.5% smaller in OVX control rats compared with intact controls. 4-OHE$_2$ at a dose of 0.05 mg/kg had no statistically significant effects on uterine epithelial cell height compared with the intact control rats. At doses of 0.5, 1.0 and 1.5 mg/kg, 4-OHE$_2$ produced a significant increase in epithelial cell height compared with the intact control rats.

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**TABLE 1**

Treatment effects on different parameters in rats from experiment 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight before surgery</th>
<th>Weight before death</th>
<th>Uterine wet weight</th>
<th>Uterus/body mass ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact control</td>
<td>222 ± 6</td>
<td>214 ± 9</td>
<td>353 ± 112$^a$</td>
<td>16 ± 5$^a$</td>
</tr>
<tr>
<td>OVX control</td>
<td>215 ± 7</td>
<td>223 ± 18</td>
<td>155 ± 13</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>OVX + E$_2$ (1 mg/kg)</td>
<td>211 ± 7</td>
<td>216 ± 12</td>
<td>367 ± 99$^a$</td>
<td>17 ± 5$^a$</td>
</tr>
<tr>
<td>OVX + 4-OHE$_2$ (1 mg/kg)</td>
<td>217 ± 12</td>
<td>214 ± 11</td>
<td>343 ± 20$^a$</td>
<td>16 ± 2$^a$</td>
</tr>
<tr>
<td>OVX + 4-OHE$_2$ (1.5 mg/kg)</td>
<td>218 ± 8</td>
<td>206 ± 20</td>
<td>339 ± 56$^a$</td>
<td>16 ± 2$^a$</td>
</tr>
<tr>
<td>OVX + E$_2$ (1 mg/kg) + TAM (1 mg/kg)</td>
<td>215 ± 9</td>
<td>206 ± 11</td>
<td>292 ± 92$^a$</td>
<td>14 ± 2$^a$</td>
</tr>
<tr>
<td>OVX + E$_2$ (1 mg/kg) + 4-OHT (0.8 mg/kg)</td>
<td>210 ± 7</td>
<td>216 ± 23</td>
<td>250 ± 47$^a$</td>
<td>12 ± 2.6$^a$</td>
</tr>
</tbody>
</table>

$^a$ Statistically different from OVX control rats (P < .05).

**TABLE 2**

Treatment effects on different parameters in rats from experiment 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight before surgery</th>
<th>Weight before death</th>
<th>Uterine wet weight</th>
<th>Uterus/body mass ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g</td>
<td>mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact control</td>
<td>221 ± 7</td>
<td>202 ± 6</td>
<td>382 ± 108$^a$</td>
<td>19 ± 6$^a$</td>
</tr>
<tr>
<td>OVX control</td>
<td>227 ± 10</td>
<td>218 ± 22</td>
<td>155 ± 14</td>
<td>7 ± 0.3</td>
</tr>
<tr>
<td>OVX + 4-OHE$_2$ (0.05 mg/kg)</td>
<td>222 ± 5</td>
<td>203 ± 26</td>
<td>216 ± 98</td>
<td>16 ± 7$^a$</td>
</tr>
<tr>
<td>OVX + 4-OHE$_2$ (0.5 mg/kg)</td>
<td>224 ± 7</td>
<td>200 ± 12</td>
<td>343 ± 37$^a$</td>
<td>17 ± 1$^a$</td>
</tr>
<tr>
<td>OVX + 4-OHE$_2$ (0.5 mg/kg) + 4-OHT (1.5 mg/kg)</td>
<td>222 ± 5</td>
<td>199 ± 9</td>
<td>239 ± 48$^a$</td>
<td>12 ± 3$^a$</td>
</tr>
<tr>
<td>OVX + 2-OHE$_2$ (0.5 mg/kg)</td>
<td>221 ± 7</td>
<td>212 ± 12</td>
<td>155 ± 10</td>
<td>7 ± 0.8</td>
</tr>
<tr>
<td>OVX + E$_2$ (0.5 mg/kg)</td>
<td>220 ± 4</td>
<td>205 ± 16</td>
<td>421 ± 145$^a$</td>
<td>21 ± 8$^a$</td>
</tr>
<tr>
<td>OVX + E$_2$ (0.5 mg/kg) + 4-OHT (1.5 mg/kg)</td>
<td>225 ± 4</td>
<td>204 ± 8</td>
<td>256 ± 44$^a$</td>
<td>13 ± 2$^a$</td>
</tr>
<tr>
<td>OVX + 2-meoE$_1$ (0.5 mg/kg)</td>
<td>227 ± 8</td>
<td>199 ± 21</td>
<td>150 ± 10</td>
<td>8 ± 1$^a$</td>
</tr>
<tr>
<td>OVX + 2-meoE$_2$ (0.5 mg/kg)</td>
<td>222 ± 9</td>
<td>200 ± 18</td>
<td>141 ± 10</td>
<td>7 ± 0.6</td>
</tr>
</tbody>
</table>

$^a$ P < .05 compared with OVX control.
### TABLE 3
Effect of different treatments on uterine wet weight and histological parameters in OVX rats
Uterine weight was divided by 100 mg before it was combined with other parameters to give the total estrogenicity score.

<table>
<thead>
<tr>
<th>Group</th>
<th>Uterine weight</th>
<th>Uterine epithelial height</th>
<th>Uterine myometrial thickness</th>
<th>Uterine stromal expansion</th>
<th>Uterine stromal eosinophilia</th>
<th>Total estrogenicity score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact control</td>
<td>382 ± 108*</td>
<td>2.2 ± 0.4*</td>
<td>3.8 ± 0.8*</td>
<td>6.4 ± 1.1*</td>
<td>3.2 ± 0.8*</td>
<td>19.4 ± 2.8*</td>
</tr>
<tr>
<td>OVX control</td>
<td>155 ± 14</td>
<td>1 ± 0</td>
<td>2.5 ± 1</td>
<td>4.5 ± 1.3</td>
<td>0.5 ± 0.6</td>
<td>10.1 ± 2.0</td>
</tr>
<tr>
<td>OVX +4-OHE2 (0.05 mg/kg)</td>
<td>216 ± 98</td>
<td>1.6 ± 1.3</td>
<td>2.8 ± 0.4</td>
<td>5.6 ± 0.5*</td>
<td>1.6 ± 2.5</td>
<td>13.8 ± 5.2</td>
</tr>
<tr>
<td>OVX +4-OHE2 (0.5 mg/kg)</td>
<td>345 ± 37*</td>
<td>2.6 ± 0.5*</td>
<td>3.0 ± 0.7</td>
<td>6.8 ± 1.1*</td>
<td>4.4 ± 1.1*</td>
<td>20.2 ± 1.9*</td>
</tr>
<tr>
<td>OVX +4-OHE2 (1.5 mg/kg)</td>
<td>345 ± 20*</td>
<td>2.8 ± 0.4*</td>
<td>2.8 ± 1.1</td>
<td>7.2 ± 1.3*</td>
<td>8.5 ± 1.3*</td>
<td>23.4 ± 2.5*</td>
</tr>
<tr>
<td>OVX +2-OHE2 (0.5 mg/kg)</td>
<td>339 ± 95*</td>
<td>3.5 ± 0.5*</td>
<td>3.6 ± 0.6</td>
<td>6.5 ± 1.3*</td>
<td>8.5 ± 1.3*</td>
<td>24.9 ± 2.1*</td>
</tr>
<tr>
<td>OVX +2-OHE2 (0.5 mg/kg) +4-OHT (1.5 mg/kg)</td>
<td>230 ± 48*</td>
<td>3.6 ± 0.5*</td>
<td>3.2 ± 0.4</td>
<td>5.8 ± 0.8*</td>
<td>2.8 ± 1.5*</td>
<td>17.8 ± 2.1*</td>
</tr>
<tr>
<td>OVX +E2 (0.5 mg/kg)</td>
<td>155 ± 10</td>
<td>1 ± 0</td>
<td>2.6 ± 0.9</td>
<td>4.6 ± 0.9</td>
<td>0.6 ± 0.9</td>
<td>10.4 ± 1.9</td>
</tr>
<tr>
<td>OVX +E2 (1 mg/kg)</td>
<td>420 ± 145*</td>
<td>3.4 ± 0.9*</td>
<td>3.2 ± 0.8</td>
<td>6.8 ± 0.8*</td>
<td>6.0 ± 3.8*</td>
<td>23.6 ± 6.7*</td>
</tr>
<tr>
<td>OVX +E2 (0.5 mg/kg)</td>
<td>367 ± 99*</td>
<td>4.0 ± 0.8*</td>
<td>4.3 ± 0.5*</td>
<td>6.0 ± 1.4</td>
<td>5.3 ± 1.1*</td>
<td>23.4 ± 1.5*</td>
</tr>
<tr>
<td>OVX +2-meoE2 (0.5 mg/kg)</td>
<td>256 ± 44*</td>
<td>3.4 ± 0.5*</td>
<td>3.4 ± 0.5*</td>
<td>5.4 ± 0.5</td>
<td>3.4 ± 1.5*</td>
<td>18.2 ± 2.2*</td>
</tr>
<tr>
<td>OVX +2-meoE2 (0.5 mg/kg)</td>
<td>150 ± 10</td>
<td>1.2 ± 0.4</td>
<td>2.6 ± 0.5</td>
<td>5.4 ± 0.5</td>
<td>0.2 ± 0.5</td>
<td>10.9 ± 1.3</td>
</tr>
<tr>
<td>OVX +2-meoE2 (0.5 mg/kg)</td>
<td>141 ± 10</td>
<td>1.2 ± 0.4</td>
<td>2.0 ± 0.0</td>
<td>5.6 ± 0.9</td>
<td>0 ± 0</td>
<td>10.2 ± 1</td>
</tr>
</tbody>
</table>

* P < .05 compared with OVX control.

Number of eosinophils per microscope field (×80).

#### Figure 2
Treatment effects on total cholesterol levels in experiment 1.
Rats were dosed po once a day for 7 days. At 24 hr after the last dose, total cholesterol was determined. Results are presented as mean ± SD.

#### Discussion
Estrogen, both natural and synthetic (e.g., ethinyl estradiol), can induce profound hypolipidemia at pharmacological doses (Davis and Robb, 1978). Certain studies on both cholesterol and reproductive tissues have reported on the beneficial effects of estrogens on the metabolism of cholesterol (Davis and Robb, 1978). Few studies have reported on the biological activities of oxygenated metabolites by using antiestrogenic substances and by creating an OVX rat model. The advantages for the use of OVX rats...
are that (1) ovariectomy can minimize the interference of endogenous estrogens, (2) ovariectomy mimics the true postmenopausal condition and (3) the model permits comparison of the simultaneous effects of oxygenated estradiol metabolites and E₂ on reproductive tissue (uterus) and TCL permitting inferences to be drawn about the role of ERs in both effects.

The results of the study reported herein are quantitatively different from the previous study in male rats. The maximal hypocholesterolemic effect (Eₘₐₓ) for 4-OHE₂ occurred at a dose of 0.05 mg/kg compared with 2.0 mg/kg in male rats. ERs are conceivably different between male and female rats both quantitatively and qualitatively, and this may contribute to the differential potencies between genders, as could differences in pharmacokinetic parameters.

In the present study, 4-OHE₂ at a dose of 0.05 mg/kg produced a significant decrease in cholesterol levels that was comparable to that elicited by E₂ at a 10 times higher dose. 4-OHE₂ also appeared to be more potent than the other three metabolites (2-OHE₂, 2-meoE₁ and 2-meoE₂). At doses of 0.5 mg/kg and higher, 4-OHE₂ virtually depleted the total cholesterol levels. It was interesting to observe that 2-OHE₂, 2-meoE₁ and 2-meoE₂ were able to significantly decrease total cholesterol levels without stimulating the uterus, since it signals that these two effects may be mediated by different mechanisms. This finding may open the door for developing new hypolipidemic drugs devoid of the side effects observed in estrogen replacement therapy. However, further study is warranted to better understand the underlying mechanisms.

It is currently proposed that ERs can exist in the cell in multiple conformations that represent the inactive state, the active state and several intermediate states and that ligands exert their biological activities by stabilizing a specific conformation. In the absence of ligand the active conformation is preferred. Interaction of ER with E₂ stabilizes the complex in a conformation that facilitates transactivation (McDonnell et al., 1995). The relative agonist/antagonist balance of other ER modulators is determined by the intermediate conformation promoted by the particular compound (McDonnell et al., 1995). The findings of this study were consistent with the notion that 4-OHT is a partial ER agonist. A number of published studies showed 4-OHT has a similar affinity for ER as that of E₂ (McDonnell et al., 1995; Osborne et al., 1992). 4-OHT and its parent drug, TAM, are classified as type IV antiestrogens, which stabilize ER in a conformation that allows it to exhibit transcriptional activity on a limited subset of ER-responsive genes (McDonnell et al., 1995). It was shown by Klinge et al. (1996) that 4-OHT-ligated ER binds the ERE DNA with high affinity, but at its saturation ERE binding capacity is consistently half that of E₂-ER, which means that one molecule of 4-OHT ligand dissociates from the ER dimer as a consequence of ERE binding. In a later separate experiment conducted in our lab, 4-OHT alone was shown to be able to produce both hypcholesterolemic and uterotrophic effects in OVX rats, which is consistent with the findings of other studies with regard to its partial ER agonism.

The results of these experiments suggest that the hypcholesterolemic effects of 4-OHE₂ are largely mediated through ER. Additional evidence is as follows: (1) partial ER antagonists partly but effectively blocked the hypcholesterolemic and uterotrophic effects of 4-OHE₂; (2) the hypcholesterolemic effect of 4-OHE₂ in OVX rats is more potent than that observed in male rats, which is likely due to more ER present in female tissues; and (3) in vitro studies showed 4-OHE₂ has an affinity for ER close to that for E₂ (Tanaka et al., 1986; Martucci and Fishman, 1976; Merrian et al., 1980; Davies et al., 1975; Schutze et al., 1993). (4) In a similar study in OVX rats, E₂ and 4-OHE₂ produced comparable levels of ER occupation in limbic brain, pituitary and uterus, and similar behavioral and gonadotrop responses were observed (Jellinck et al., 1981).

That the hypcholesterolemic effect of estrogen metabolites can be at least partially dissociated from ERs is suggested by the following: First, among these four E₂ metabolites, 4-OHE₂ has the strongest affinity for ER, followed by 2-OHE₂, whereas the methylated products of 2-OHE₂ and 2-OHE₁, 2-meoE₂ and 2-meoE₁, respectively, virtually have no affinity for ER (Martucci and Fishman, 1976; Merrian et al., 1980) yet they retain significant, although smaller, hypcholesterolemic effects. Thus, it may be inappropriate to attribute the hypcholesterolemic effects of 4-OHE₂, 2-OHE₂, 2-meoE₂ and 2-meoE₁ solely to the ligand-ER interactions. Second, 4-OHE₂ is a much more potent hypcholesterolemic agent than E₂, even though they have similar affinity to ER. Third, both 2-OHE₂ and 4-OHE₂ are eliminated from the body at a much faster rate than E₂ with metabolic clearance rates in an apparent ratio of 1:4:11 (E₂/4-OHE₂/2-OHE₂) (Ball et al., 1983; Emons et al., 1982). This pharmacokinetic
property would be expected to diminish the apparent potency of 4-OHE\textsubscript{2} relative to E\textsubscript{2} because one would expect lower tissue concentrations of 4-OHE\textsubscript{2} than for E\textsubscript{2} when the same dose is administered. Finally, nonsteroidal compounds, such as TAM and benzofurans, can bind to another class of intracellular binding sites, often termed “antiestrogen binding sites” or “AEBS,” which do not bind E\textsubscript{2} (Lazier and Bapat, 1988; Teo \textit{et al.}, 1992). It has been shown that selective ligands of AEBS are very likely to be involved in the inhibition of de novo cholesterol biosynthesis in cell culture (Teo \textit{et al.}, 1992; Cypriani \textit{et al.}, 1988). One interpretation of our findings is that non-ER-based mechanisms may play a role in the cholesterol-lowering effect of the oxygenated estrogen metabolites, and this would be consistent with a possible role at non-ER binding sites. It is well known that estrogen can lower cholesterol levels through up-regulating LDL receptors on the hepatic cell surface by acting at ER and subsequently accelerating the plasma clearance of cholesterol. The cholesterol synthetic pathway is another potential site of action for estrogen metabolites. There are several steps in the synthesis of cholesterol, each catalyzed by enzymes, which could be inhibited by estrogen metabolites in a manner akin to the inhibition of HMG COA reductase by lovastatin. Similarly, cholesterol catabolism in the liver could also be expedited by the stimulation of enzymes that convert cholesterol into bile acids. There is no proof for these hypotheses, and further investigations are warranted.

Uterine wet weight has long been used as a reliable parameter in evaluating the uterotrophic effects of certain estrogenic compounds (Levin \textit{et al.}, 1968; Levin \textit{et al.}, 1967). The recent application of histological markers makes the evaluation a more convincing one (Black \textit{et al.}, 1994). In this study, 2-OHE\textsubscript{2}, 2-meo\textsubscript{E}_{1} and 2-meo\textsubscript{E}_{2} showed no uterotropic activity, but they significantly decreased cholesterol levels, albeit not as much as E\textsubscript{2} or 4-OHE\textsubscript{2}. It is conceivable that pharmacokinetic differences between these three compounds could contribute to these observations. In addition, 2-meo\textsubscript{E}_{1} and 2-meo\textsubscript{E}_{2} can undergo demethylation and produce corresponding 2-hydroxyestrogens (Martucci and Fishman, 1993), which may actually mediate the hypocholesterolemic effect of 2-meo\textsubscript{E}_{1} and 2-meo\textsubscript{E}_{2}. However, it is equally likely that these three oxygenated estrogen metabolites may exert their
hypocholesterolemic effect via an ER-independent pathway, in a manner analogous to the inhibition of de novo cholesterol synthesis by benzofturans in cells which lack ER (Teo et al., 1992). This hypothesis is supported by the finding that 2-mEO2 inhibits tubulin polymerization by acting at an ER-independent colchicine site, thereby inhibiting angiogenesis and breast cancer in mice (Klauber et al., 1997). Additionally, Joselsson and Tarkowski (1997) reported that 2-mEO2 can suppress angiogenesis without showing feminizing effects on sex organs. In light of the serious human health consequences of coronary heart disease, the implication that substances related to 2-OHE2, 2-mEO2, and 2-mEO2 might offer a useful therapy for postmenopausal women to maintain lower serum cholesterol without affecting reproductive tissue merits further investigation.

Finally, the different roles of the two ER subtypes, ERα and ERβ, warrant brief comment. ERα refers to the classic ER. ERβ was cloned in 1995 (Kuiper et al., 1996) and has since been found to exist in a number of tissues in both humans and animals (Mosselman et al., 1996; Arts et al., 1997). A recent study showed that ERα and ERβ signal in opposite ways when complexed with the ER from an activator protein-1 site. ERα-ERβ activated transcription, whereas ERβ inhibited transcription. Moreover, TAM, raloxifene, and ICI 164384 were shown to be potent transcriptional activators with ERβ at an activator protein-1 site. Thus, the two ERs signal in different ways depending on the ligand and response element (Paech et al., 1997). The difference in the distribution densities of these two ER subtypes may also partly explain the different pharmacological responses we observed in this study. Characterization of the distribution of each ER subtype in uterus and liver is warranted.

In conclusion, the present study on estradiol metabolites has provided evidence that oxygenated estradiol metabolites possess hypocholesterolemic activities that can be separated, in part, from their uterotrophic effects in ovariectomized rats.

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