Differential Effects of Oral and Transdermal Estrogen/Progesterone Regimens on Sensitivity to Activated Protein C Among Postmenopausal Women

A Randomized Trial

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Objective—Activated protein C (APC) resistance not related to the factor V Leiden mutation is a risk factor for venous thrombosis. Oral estrogen replacement therapy (ERT) has been reported to induce APC resistance. Little is known about the effect of transdermal estrogen.

Methods and Results—We enrolled 196 postmenopausal women who were randomly allocated to receive either 1 mg 17β-estradiol orally (n=63) or 50 μg 17β-estradiol transdermally per day (n=68), both associated with 100 mg progesterone daily or placebo (n=65) for 6 months. An activated partial thromboplastin time (APTT)–based test and the effect of APC on thrombin potential (ETP) were used. Oral ERT induced an ETP-based APC resistance compared with both placebo (P=0.006) and transdermal ERT (P<0.001), but there was no significant effect of transdermal ERT compared with placebo (P=0.191). There was no significant effect of ERT on the APTT-based APC sensitivity ratio. Prothrombin fragment 1+2 plasma levels were significantly higher after 6 months of treatment in women allocated to oral ERT compared with those on placebo and transdermal ERT and were positively and significantly correlated with changes in ETP-based APC sensitivity ratio.

Conclusions—Our data show that oral, unlike transdermal, estrogen induces APC resistance and activates blood coagulation. These results emphasize the importance of the route of estrogen administration. (Arterioscler Thromb Vasc Biol. 2003;23:1671-1676.)

Key Words: hormone replacement therapy ■ APC resistance ■ blood coagulation ■ randomized trial ■ factor V

Several observational studies1–7 found that oral estrogen replacement therapy (ERT) was associated with a 2-fold increased risk of venous thromboembolism (VTE). This finding was confirmed in 2 randomized clinical trials.8,9 Furthermore, consistent data provided evidence that oral ERT resulted in coagulation activation.10–14 However, studies investigating the effect of transdermal estrogen on the thrombotic process are scarce.3,15

Activated protein C (APC) resistance has recently emerged as a risk factor for venous thrombosis.16,17 In most cases, this defect is related to the presence of the R506Q mutation in factor V (FV) Leiden.18 However, an APC-resistant phenotype detected in the absence of FV Leiden is also an independent risk factor for venous thrombosis.19 Observational studies20–22 and 1 randomized trial23 showed that oral ERT could induce an acquired APC resistance. Little is known about the effect of transdermal ERT on APC resistance. Therefore, we conducted a randomized, placebo-controlled trial that investigated the effect of both oral and transdermal estrogen/progesterone regimens on the anticoagulant response to APC and on coagulation activation.

Methods

Study Design and Setting
This study was a randomized, double-blind, placebo-controlled, parallel-group trial that took place at the Hôpital de la Cavale Blanche, Brest, France, between September 1999 and August 2001. Participants were followed up by regular visits, at randomization (baseline), and after 6 months. A medical review with use of a standardized questionnaire was conducted at baseline, and a medical examination was performed at each visit.

Subjects
Alltogether, 196 postmenopausal women younger than 70 years were randomized. Postmenopausal was defined as follows: no natural...
menstruation for at least 6 months, no progestrone-induced menstruation over 3 cycles (10 days on treatment followed by 18 days off), or bilateral ovariectomy or hysterectomy with concentrations of follicle-stimulating hormone >40 IU/mL and estradiol <20 pg/mL. A normal mammography result was also required. Exclusion criteria were the following: gynecological cancer, cardiovascular disease (heart valve disease, coronary heart disease, stroke, atrial fibrillation, or uncontrolled arterial hypertension), previous venous thrombosis, and liver insufficiency. Women who had used hormone replacement therapy (HRT) or oral contraceptives in the previous 3 months were excluded, as were women on antithrombotic treatment. Women were recruited through gynecologist and endocrinologist practitioners (list in Appendix) in the Brest area. The protocol was approved by the local ethics committee. Written, informed consent was obtained from all women. The study was carried out according to the Helsinki Declaration and Good Clinical Practice.

Interventions

The participants were allocated to 1 of the following 3 groups: (1) 1 mg/d of oral 17β-estradiol, (2) 50 μg/d transdermal 17β-estradiol, both combined with 100 mg/d oral, micronized progesterone on a continuous basis, or (3) triple-dummy, equal-looking placebo. The allocation schedule was computer generated with a random block size-stratified according to the recruitment procedure (endocrinologists or gynecologists). The Cavale Blanche Hospital Pharmacy Department had the randomization list and received from the manufacturers blister packs containing pills, capsules, and patches of either active drugs or placebo, identical in appearance. The blister packs were supplied in sequentially numbered, tamper-proof, and similar-looking containers. The Pharmacy Department distributed the containers and retained the trial codes, which were disclosed after the study. The participants, investigators, and outcome assessors remained unaware of the intervention assignment throughout the trial. The women were requested to take tablets at bedtime, and a continuous regimen was chosen to avoid regular bleeding in the 2 active treatment groups; thus, strong hints were minimized.

Blood Collection

Blood samples were drawn between 8 and 10 AM after an overnight fast and a 10-minute rest. With regard to coagulation measurements, venous blood (9 volumes) was collected in 5-mL evacuated tubes (Vacutainer, Becton-Dickinson) containing 0.11 mol/L/L trisodium citrate (1 volume). Platelet-poor plasma was obtained by 2 centrifugation steps at 2500 rpm for 15 minutes at 15°C. Aliquots were transferred to plastic tubes, quickly frozen, and stored at −80°C. Venous blood was also collected in tubes containing EDTA (0.084 mL K<sub>3</sub>EDTA for 7 mL blood) for DNA extraction and in plain tubes for hormonal measurements.

Laboratory Investigations

At the time of assay, plasma samples were transferred to a 37°C water bath for 5 minutes and then handled at room temperature. Baseline and 6-month samples from the same subject were analyzed in the same batch. The activated partial thromboplastin time (APTT)-based APC resistance assay was performed on an ST888 analyzer (Diagnostica Stago) with use of a commercially available kit (Coatest APC resistance, Biogenics). The response to APC was also determined with a 1-step immunometric chemoluminescent assay on an Immulite (Dade Behring). The free (FPS) and total (TPS) protein S antigen levels and the free (FTFPI) tissue pathway inhibitor levels were also measured with a commercially available kit (Asserachrom kits from Diagnostica Stago).

Other Laboratory Measurements

High-molecular-weight DNA was isolated from lymphocytes by phenol-chloroform extraction. Genotyping for the FV Leiden mutation was performed as previously described. Serum estradiol was quantified with an enzyme-linked fluorescent assay (VIDAS Estradiol II, Biomérieux). Serum follicle-stimulating hormone was determined with a 1-step immunometric chemoluminescent assay on an automated system (VITROS Eci, Ortho-Clinical Diagnostics).

Sample Size Estimation

With a 5% 2-sided α level, 60 subjects per group showed a difference between groups of about two thirds SD for a normally distributed variable with 95% statistical power.

Statistical Analysis

Data are presented as mean and SD. Treatment effects were calculated as the change in APCsr from baseline by ANOVA. Pairwise differences were assessed with a post hoc ANOVA with Bonferroni adjustment. Variables with skewed distributions were logarithmically transformed. Owing to the mechanistic study objective, a per-protocol rather than an intention-to-treat analysis was performed. A 2-sided value of P < 0.05 was considered statistically significant. The Spearman coefficient correlation was used to detect any association between changes in APCsr and changes in hemostatic variables. All statistical analyses were performed with the SPSS 10.0 statistical software package (SPSS for Windows, SPSS Inc.).

Results

Figure 1 shows the trial profile. Altogether, 196 women were enrolled between September 7, 1999, and August 20, 2001: 63 were allocated to receive oral estradiol plus oral progesterone, 68 to receive transdermal estradiol plus oral progesterone, and 65 to receive placebo. Thirty-six women (18.4%) discontinued their treatment because of vaginal bleeding or mastodynia (5 women in the oral group, 2 women in the transdermal group), hot flashes...
(3 women in the placebo group), or miscellaneous reasons (skin problem caused by an allergy, edema, headache, or nonreported reasons). Among these 36 women, 29 (14.8%) were lost to follow-up at 6 months and 7 attended the final visit. Overall, 167 women attended the 6-month visit, and 160 women completed the trial with effective intervention.

Table 1 shows the baseline characteristics of participants by treatment group. Women ranged in age from 43 to 69 years, with a mean age of 53.2 years at baseline. There was no significant imbalance between the groups with regard to age and cardiovascular risk factors (hypertension, diabetes, lipid profile, tobacco use, and body mass index). Seventeen women (8.7%) carried the FV Leiden mutation. Table 2 shows the changes in sex hormone levels by treatment group. Both active treatments significantly increased plasma estradiol levels and decreased follicle-stimulating hormone levels. Levels of both sex hormones remained unchanged in the placebo group.

According to the per-protocol analysis scheme, we analyzed only the 160 women who completed the trial and actually took the study drug. Table 3 shows values of hemostatic variables at baseline and after 6 months of follow-up, as well as mean changes by treatment group. Oral ERT significantly increased the ETP-based nAPCsr, ie, induced an APC resistance, compared with both placebo \((P=0.006)\) and transdermal estrogen \((P<0.001)\). Transdermal ERT had no significant effect on the endogenous thrombin potential (ETP)-based nAPCsr compared with placebo \((P=0.191)\). ERT, either oral or transdermal, had no significant effect on the APTT-based APCsr. Similar results were found when the analysis was restricted to FV Leiden noncarriers (data not shown).

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**TABLE 1. Baseline Characteristics of Participants**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Oral Estradiol + Progesterone n=63</th>
<th>Estradiol Patch + Progesterone n=68</th>
<th>Placebo n=65</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, y</strong></td>
<td>53±4</td>
<td>52±4</td>
<td>54±4</td>
</tr>
<tr>
<td><strong>Duration of amenorrhea, months</strong></td>
<td>37±59</td>
<td>30±36</td>
<td>40±55</td>
</tr>
<tr>
<td><strong>Known and treated hypertension, n (%)</strong></td>
<td>7 (11.1)</td>
<td>10 (14.7)</td>
<td>12 (18.5)</td>
</tr>
<tr>
<td><strong>Systolic BP, mm Hg†</strong></td>
<td>124±14</td>
<td>124±19</td>
<td>125±19</td>
</tr>
<tr>
<td><strong>Diastolic BP, mm Hg†</strong></td>
<td>72±11</td>
<td>74±11</td>
<td>73±9</td>
</tr>
<tr>
<td><strong>Diabetes, n (%)‡</strong></td>
<td>2 (3.2)</td>
<td>4 (5.9)</td>
<td>8 (12.3)</td>
</tr>
<tr>
<td><strong>Abnormal lipid profile, n (%)§</strong></td>
<td>6 (9.5)</td>
<td>5 (7.3)</td>
<td>8 (12.3)</td>
</tr>
<tr>
<td><strong>Current smoker, n (%)</strong></td>
<td>8 (12.7)</td>
<td>13 (19.1)</td>
<td>14 (21.6)</td>
</tr>
<tr>
<td><strong>Ever smoker, n (%)</strong></td>
<td>10 (15.9)</td>
<td>17 (25.0)</td>
<td>9 (13.8)</td>
</tr>
<tr>
<td><strong>Never smoker, n (%)</strong></td>
<td>45 (71.4)</td>
<td>38 (55.9)</td>
<td>42 (64.6)</td>
</tr>
<tr>
<td><strong>Body mass index, kg/m²</strong></td>
<td>24.4±3.5</td>
<td>24.9±4.3</td>
<td>24.8±4.6</td>
</tr>
<tr>
<td><strong>Factor V Leiden carriers, n (%)</strong></td>
<td>7 (11.1)</td>
<td>6 (8.8)</td>
<td>4 (6.2)</td>
</tr>
</tbody>
</table>

Values are mean±SD or number (percentage).

*Hypertension includes women having a systolic blood pressure \(\geq 140\) mm Hg or a diastolic blood pressure \(\geq 90\) mm Hg at baseline and at 3 months, or women who were receiving antihypertensive drugs at baseline.

†In women not taking antihypertensive drugs.

‡Diabetes includes women having a baseline plasma glucose level \(\geq 126\) mg/dL or women who were taking insulin or receiving oral treatment for diabetes.

§Abnormal lipid profile includes women having a baseline LDL cholesterol level \(\geq 130\) mg/dL or a triglycerides level \(\geq 200\) mg/dL or women who were receiving lipid-lowering drugs.
ERT significantly decreased TPS levels compared with placebo. Oral and transdermal ERT had no significant effect on TTFPI and FPS levels. Changes in the ETP-based nAPCsr were not significantly correlated with changes in either TTFPI or TPS levels in FV Leiden noncarriers allocated to oral ERT. The intention-to-treat analysis scheme showed similar results.

**Discussion**

The main finding of this randomized trial is that oral, unlike transdermal, ERT (1 mg orally or 50 μg transdermally 17β-estradiol, both plus 100 mg oral, micronized progesterone, given on a continuous basis over 6 months) significantly alters the effect of APC on thrombin generation (ETP-based assay), leading to an acquired APC resistance. Furthermore, the oral estrogen/progesterone treatment was associated with significantly higher plasma prothrombin fragment 1+2 levels, a marker for coagulation activity, than placebo or transdermal treatment, with changes in prothrombin fragment 1+2 levels being correlated with changes in the ETP-based nAPCsr.

Four randomized, placebo-controlled trials previously investigated the effect of ERT on APC resistance. All of these trials studied oral ERT but used different methods to measure APC resistance: the original APTT-based assay, the Staclot APC-R test, and the ETP-based nAPCsr in the last 1.2, 13, 14 In the first 2 trials, a thrombin generation assay was used in the third trial, 15 and the Staclot APC-R test in the last 1.16 In the first 2 trials, oral ERT had no significant effect on the APTT-based APC resistance at 3 months. Our data are consistent with these previous findings. However, in agreement with several reports, we confirm that oral ERT significantly decreases TPS but does not change the FPS level.

The safety of HRT with regard to VTE is an important issue. Early studies of VTE risk among ERT users provided inconclusive results. 2 More recently, observational studies showed consistent associations between current use of ERT and risk of VTE in postmenopausal women. 17-21 These results have been clearly confirmed by randomized clinical trials. 3-5 However, most of those studies investigated women who were using conjugated equine estrogens alone or combined with progestin. These results do not apply to users of transdermal ERT. Clinical data evaluating the influence of the route of estrogen administration are scarce. Two case-control studies reported no difference in VTE risk between users of oral and transdermal ERT. 4,5 However, those results were based on 5 and 7 cases of VTE exposed to transdermal estrogen, and confidence intervals were wide. Therefore, data on the effect of transdermal estrogen on VTE risk remained inconclusive.

Biologic evidence supports a differential effect of oral versus transdermal estrogen on hemostasis. Randomized trials have shown that oral ERT increases plasma levels of prothrombin fragment F1+2, 22 which is a marker for in vivo thrombin generation and which was recently related to the risk of recurrent VTE. 9 Consistent data reported that transdermal ERT had no detrimental effect on coagulation. 11, 23, 25 Especially prothrombin fragment 1+2 plasma level, and our findings are in accordance with these results. Thus, oral ERT might impair the balance between procoagulant factors and antithrombotic mechanisms, whereas transdermal ERT appears to have little or no effect on hemostasis.

APC resistance has recently emerged as a risk factor for VTE. 16, 17 Although this phenotype is mostly related to the FV Leiden mutation, 18 APC resistance detected in the absence of

### Table 2. Plasma Estradiol (E2) and FSH at Baseline and After 6 Months by Treatment Groups (Oral 17 β-Estradiol [E2] 1 mg or Transdermal 17 β-Estradiol [E2] 50 μg, Both Combined With Micronized Progesterone [P] 100 mg/d Continuously)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Treatment</th>
<th>No.</th>
<th>Baseline</th>
<th>After 6 Months</th>
<th>Difference</th>
<th>P†</th>
<th>P‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH, IU/mL</td>
<td>Placebo</td>
<td>54</td>
<td>63±24</td>
<td>64±27</td>
<td>2±18</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E2 oral +P</td>
<td>55</td>
<td>62±29</td>
<td>48±23</td>
<td>−31±110</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E2 patch +P</td>
<td>58</td>
<td>55±29</td>
<td>43±23</td>
<td>−15±24</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2, pg/mL</td>
<td>Placebo</td>
<td>54</td>
<td>42±98</td>
<td>43±47</td>
<td>−3±83</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>E2 oral +P</td>
<td>55</td>
<td>51±74</td>
<td>193±133</td>
<td>140±131</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>E2 patch +P</td>
<td>58</td>
<td>47±41</td>
<td>70±63</td>
<td>25±68</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SD.

*Analysis of variance.

†Comparison between active treatment and placebo.

‡Comparison between the two active treatments.

Statistical tests were performed after logarithmic transformation of the variables.

mg conjugated equine estrogen plus 5 mg medroxyprogesterone acetate over 6 months resulted in an acquired APC resistance. The mechanistic basis of APC resistance induced by oral treatment is not clear. It has been reported that changes in FPS and TFPI might have important roles in determining the sensitivity of plasma to APC. Our results do not confirm these previous findings. However, in agreement with several reports, we confirm that oral ERT significantly decreases TPS but does not change the FPS level.

The main finding of this randomized trial is that oral, unlike transdermal, ERT (1 mg orally or 50 μg transdermally 17β-estradiol, both plus 100 mg oral, micronized progesterone, given on a continuous basis over 6 months) significantly alters the effect of APC on thrombin generation (ETP-based assay), leading to an acquired APC resistance. Furthermore, the oral estrogen/progesterone treatment was associated with significantly higher plasma prothrombin fragment 1+2 levels, a marker for coagulation activity, than placebo or transdermal treatment, with changes in prothrombin fragment 1+2 levels being correlated with changes in the ETP-based nAPCsr.
any thrombogenic mutation has also been shown to be an independent risk factor for VTE. Oral ERT alters APC resistance as measured by a thrombin generation assay; moreover, our data exhibited a correlation between prothrombin fragment 1+2 level and the ETP-based APCsr. Taken together, these data provide a plausible biologic mechanism to the clearly demonstrated association between oral estrogen and venous thrombosis. In addition, our data provide further evidence that transdermal ERT has little or no effect on APC resistance. This result extends and strongly supports findings from observational and nonrandomized studies. There is now a strong body of biologic evidence that suggests a lower risk of VTE, if any, among users of transdermal ERT. However, these trials investigated the effect of transdermal ERT on biologic markers, and none used clinical end points. Whether transdermal ERT is a safe option for the relief of severe climacteric symptoms needs further investigation that our study might well stimulate.

In summary, our data show that oral, unlike transdermal, ERT induces an acquired APC resistance and activates blood coagulation. These results underline the potential importance of the route of estrogen administration in prescribing HRT.

**SARAH Investigators**

The SARAH investigators are listed in alphabetical order for each group. Steering committee: Michel Collet, Véronique Kerlan, Dominique Mottier, Emmanuel Oger (project coordinator), and Pierre-Yves Scarabin; Writing committee: Martine Alhenc-Gelas, Emmanuel Oger, and Pierre-Yves Scarabin; Project management: Ghislaine Kermagoret and Karine Lacut (associate project coordinator); Investigators: Jean Ahaq, Florence Cariou, Michèle Chanier, Armelle Cozic, Philippe Dannequin, François-Xavier Gayet, Sylvana Ghezzi-Sybille, Henri Gouëd, Alain Hassoun, Claude Lacarce, Hubert Le Bos, Rémi Le Bourdonnec, Bernard Letellier, Nathalie Roudaut, Gilles Salnelle, Catherine Thomas, Jean-Jacques Turlan, Nadine Touffet, and Nesca Witwoet.

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References


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