Postmenopausal Hormone Replacement Therapy Increases Coagulation Activity and Fibrinolysis

Helena J. Teede, Barry P. McGrath, Joseph J. Smolich, Erica Malan, Dimitra Kotsopoulos, Yu-Lu Liang, Roger E. Peverill

Abstract—Hormone replacement therapy (HRT) appears to be cardioprotective in postmenopausal women; however, concerns exist over its thrombogenic effects. To address the effects of combined HRT on coagulation and fibrinolysis, we have measured circulating markers of these processes in a double-blind placebo-controlled trial. Forty-two healthy postmenopausal women aged 50 to 75 years received continuous combined HRT with 2 mg estradiol +1 mg norethisterone or placebo for 6 weeks. Hormone profiles were measured at baseline, and lipid and hemostatic parameters were measured at baseline and after 6 weeks of therapy. Baseline characteristics were similar in the 2 groups. With change from baseline the main outcome measure, HRT increased the markers of coagulation (prothrombin fragments 1+2, 0.20±0.06 versus 0.06±0.04 nmol/L, P=0.0005; soluble fibrin, 2.3±0.4 versus 0.25±0.3 µg/mL, P=0.0004), reduced plasma fibrinolytic inhibitory activity (plasminogen activator inhibitor-1, −0.67±0.16 versus 0.24±0.21 U/mL, P=0.002), and increased fibrinolysis (D-dimer, 24±12 versus −6±8 ng/mL, P=0.04) compared with placebo. Increases in soluble fibrin and D-dimer were positively correlated (r=0.59, P=0.02), but changes in plasminogen activator inhibitor-1 and D-dimer were unrelated. Although baseline hemostatic and lipid parameters were correlated, there were no associations between changes in hemostatic markers and lipids after treatment. Short-term oral combined continuous HRT (estriodol and norethisterone) increased thrombin and fibrin generation, reduced plasma fibrinolytic inhibitory activity, and increased fibrinolysis. Enhanced fibrinolysis was related to increased fibrin generation but not reduced plasma fibrinolytic inhibitory activity. Coagulation activation may partly explain the increases in venous thrombosis and cardiovascular events reported with the use of combined HRT. (Arterioscler Thromb Vasc Biol. 2000;20:1404-1409.)

Key Words: thrombosis ■ coagulation ■ fibrinolysis ■ hormone replacement therapy ■ hemostatic markers

Postmenopausal hormone replacement therapy (HRT) reduces menopausal symptoms and protects against osteoporosis.1 Epidemiological studies have also consistently suggested that postmenopausal women using HRT have a reduced risk of coronary artery disease.2,3 This reduced risk of coronary events is believed to be mediated by an inhibition of the atherosclerotic process, a hypothesis that is consistent with the beneficial effects of HRT in humans on plasma lipids4 and supported by animal studies showing that HRT reduces the severity of atherosclerosis.5,6 However, thrombosis is also an important component of coronary events, and concerns have been raised about the potential thrombogenic effects of estrogen in the venous and arterial vasculature. High-dose estrogen elevates the incidence of myocardial infarction in men,7 whereas HRT in standard doses may increase myocardial infarction and unstable angina in women with significant preexisting atherosclerosis8,9 and has also been associated with an increased incidence of venous thromboembolism.8,10,11

Thrombosis results from inappropriate initiation and propagation of the hemostatic response and may occur as a result of activation of coagulation or inhibition of fibrinolysis.12 That estrogen has a complex effect on the mechanisms underlying thrombosis is suggested by clinical studies indicating that although it produces a dose-dependent increase in plasma markers of thrombin and fibrin generation,13,14 it also decreases plasma fibrinolytic inhibitory activity14-18 by decreasing levels of plasminogen activator inhibitor-1 (PAI-1).14,16-18 However, there is considerable uncertainty regarding the effects of combined HRT with estrogen and progestin on coagulation and fibrinolytic processes. First, findings concerning the effects of combined HRT on coagulation activation have been variable, with reports of an increase in thrombin generation19,20 as well as no change in thrombin generation16,21 or fibrin production.16 Second, the effect of combined HRT on fibrinolysis is also controversial, with reports of normal20 and increased18 levels of D-dimer, a specific marker of fibrinolysis. Last, although elevation of...
D-dimer levels with combined HRT has been considered to reflect increased fibrinolysis secondary to reduced PAI-1 activity, an alternate possibility is that it may also be due to a reactive increase in fibrinolysis arising from a rise in fibrin production accompanying the activation of coagulation processes. However, this possibility has not been explored because changes in fibrin production and fibrinolysis have not been simultaneously assessed in previous studies of combined HRT therapy on hemostatic processes.

Accordingly, the aim of this double-blind, randomized, placebo-controlled study was to determine the hemostatic effects of 6 weeks of treatment of oral combined HRT treatment with estradiol and norethisterone in healthy postmenopausal women. Thrombin generation was assessed by measuring plasma levels of prothrombin fragments 1+2 (F1+2); fibrin production, by plasma levels of soluble fibrin; fibrinolysis, by plasma levels of D-dimer; and plasma fibrinolytic inhibition, by PAI-1 activity. In view of previous evidence of a relation between cardiovascular risk factors and hemostatic markers in other patient populations, the relation between hemostatic markers and lipids before and after treatment with HRT was also evaluated.

### Methods

#### Study Design

Forty-two healthy postmenopausal women were enrolled in a double-blind, placebo-controlled, randomized trial conducted over 6 weeks. Participants were aged 50 to 75 years, had not received any hormone replacement therapy for 12 months, and had no history of hysterectomy or oophorectomy. Postmenopausal status was defined as absence of menses for at least 12 months, a follicle-stimulating hormone level >23 IU/L, and estradiol <120 pmol/L. Women were enrolled from the community after responding to an advertisement in community newspapers. Exclusion criteria included smoking within the past 10 years, a history of diabetes, body weight >100 kg, alcohol or illicit drug abuse, uncontrolled hypertension (blood pressure >180/100 mm Hg), history of venous thrombosis, breast or endometrial cancer, abnormal uterine bleeding, abnormal cervical smear or mammogram results, or the presence of a major illness. Randomization was performed by an experienced researcher not otherwise involved in the study who used computer-generated random numbers, which resulted in the assignment of 22 women to HRT and 20 to placebo. Women began either continuous combined therapy with oral estradiol (2 mg) combined with continuous norethisterone acetate (1 mg, Klogest, Novo-Nordisk) or placebo tablets, having an identical appearance.

A baseline medical review was conducted with cardiovascular risk factor assessment, medication details, breast examination, and cervical smears and screening mammograms if indicated. Medical follow-up was initiated at the 6-week visit with compliance checked by counting returned tablets and by questionnaire. Ethics approval was obtained from the Monash Medical Center Human Research and Ethics Committee, and all participants gave written informed consent before their enrollment in the study. These women represented a consecutively recruited subgroup enrolled in an ongoing study of HRT and cardiovascular disease. Two withdrawals occurred in the active therapy group because of vaginal bleeding, and 1 withdrawal occurred because of unforeseen overseas travel by the participant, leaving 39 women who completed the entire study protocol. Compliance in the women who completed the study averaged 98%, with the least compliant individual consuming 88% of medications provided.

#### Blood Collection

Gonadotropins and serum estradiol were measured at baseline. Fasting morning blood samples were collected via nontraumatic phlebotomy by a single technician (D.K.) at baseline and at 6 weeks for lipid profiles and hemostatic parameters. Venipuncture was performed with a 19-gauge needle; samples were placed directly into plain tubes for lipid and hormone assays and then into 2 3.8% citrate tubes (9:1 ratio) for coagulation and fibrinolysis studies. After immediate centrifugation of samples at 2500g for 12 minutes, plasma was separated into 200 μL aliquots, stored at −80°C, and thawed immediately before analysis.

#### Hormone and Lipoprotein Assays

Gonadotropins and estradiol were measured by radioimmunoassay. Total cholesterol and triglycerides were measured by using enzymatic reagents (DADE Diagnostics); HDL cholesterol (HDL-C) was measured by homogeneous HDL-C assay techniques (HDL-Plus, DADE Diagnostics) adapted to a DADE Dimension RXL chemistry analyzer (DADE Diagnostics); and LDL cholesterol (LDL-C) was calculated by using the Friedewald equation. LDL-C=(total cholesterol−HDL-C)−(triglycerides/5.2), adapted to SI units.

#### Hemostatic Markers

All hemostatic markers were assayed by a single trained medical scientist (E.M.), with all assays performed in duplicate and the results averaged. All samples from each individual participant were assayed in the same batch. Commercial immunoassays were used to measure concentrations of F1+2 (Enzygnost F1+2, Behring), soluble fibrin (Enzymum FM, Boehringer-Mannheim) on an ES300 instrument (Boehringer-Mannheim), PAI-1 activity (Berichrom PAI, Behring), and D-dimer (Dimertest Gold EIA, Agen Biomedical).

#### Statistics

Statistical calculations were performed by using the SPSS statistical package, version 8. Assessments of treatment effects were based on the mean change from baseline. Data from all participants was included in the analysis, with the exception of 2 samples with soluble fibrin and D-dimer levels >10 SD above the mean, which were excluded because of presumed activation occurring during blood collection. Data were normally distributed, except for levels of D-dimer, which have been expressed as median (25th to 75th percentile). Change in D-dimer was analyzed by a Mann-Whitney U test, and comparison of within-group treatment effects on D-dimer was analyzed with a Wilcoxon signed rank test. Other parameters have been expressed as mean±SE and were analyzed by a Student r test. Univariate analyses were calculated by Pearson (parametric data) or Spearman (nonparametric data) correlation coefficients. Significance was accepted at a level of P<0.05.

### Results

#### Baseline Hormone, Lipid, and Hemostatic Parameters

Estradiol levels were below detectable limits in most women, and all women had gonadotropin levels consistent with their postmenopausal state (Table 1). The HRT and placebo groups...
TABLE 2. Lipid Profiles at Baseline and 6 wk: HRT vs Placebo

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Time Point</th>
<th>HRT</th>
<th>Placebo</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>Baseline</td>
<td>5.9±0.2</td>
<td>6.0±0.2</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>6 wk</td>
<td>5.2±0.2</td>
<td>5.8±0.2</td>
<td>0.03</td>
</tr>
<tr>
<td>LDL-C, mmol/L</td>
<td>Baseline</td>
<td>3.8±0.2</td>
<td>4.0±0.2</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>6 wk</td>
<td>3.4±0.2</td>
<td>3.8±0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>HDL-C, mmol/L</td>
<td>Baseline</td>
<td>1.6±0.1</td>
<td>1.5±0.1</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>6 wk</td>
<td>1.4±0.1</td>
<td>1.5±0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>Baseline</td>
<td>1.1±0.1</td>
<td>1.2±0.2</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>6 wk</td>
<td>1.1±0.1</td>
<td>1.1±0.2</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

were well matched for baseline characteristics, including lipid profiles (Tables 1 and 2) and levels of hemostatic markers (Table 3). Univariate analysis of the relations between hemostatic parameters and cardiovascular risk factors indicated that F1+2 was positively correlated with age (r=0.47, P=0.002), whereas soluble fibrin was positively correlated with body mass index (BMI; r=0.51, P=0.001) and negatively correlated with HDL-C (r=−0.35, P=0.03). D-dimer was negatively correlated with HDL-C (r=−0.37, P=0.02) and showed a trend toward a positive correlation with LDL-C (r=0.31, P=0.05), whereas PAI-1 was positively correlated with triglycerides (r=0.61, P=0.0001) and BMI (r=0.37, P=0.02) and negatively correlated with age (r=−0.46, P=0.002) and HDL-C (r=−0.42, P=0.009). Total cholesterol levels did not correlate with any hemostatic parameters.

Assessment of relations between baseline levels of fibrin and fibrinolytic markers indicated that the soluble fibrin level was positively correlated with the D-dimer level (r=0.34, P=0.03); however, no relation was evident between the PAI-1 activity and D-dimer levels.

Effects of HRT on Lipid and Hemostatic Parameters

No patient within this study had clinical evidence of thrombosis during the treatment period. After 6 weeks of treatment, total cholesterol levels were lower in the group treated with HRT compared with placebo, but there were no significant differences in the other lipid parameters (Table 2). Table 3 gives the values of hemostatic markers by treatment group at baseline and after 6 weeks of treatment. In the HRT group, levels of F1+2, soluble fibrin, and D-dimer increased significantly by ~20%, 75%, and 100%, respectively, whereas there was a 25% fall in PAI-1 activity. In contrast, no significant changes were observed in any of the hemostatic parameters in the placebo group. The mean change from baseline was significantly greater in the HRT group than in the placebo group for F1+2, soluble fibrin, D-dimer, and PAI-1 (Figure 1).

In the HRT group, there were no observed correlations between changes in lipid and hemostatic parameters. There was a positive correlation between the increase in soluble fibrin and D-dimer levels (r=0.59, P=0.02; Figure 2) but no correlation evident between the decrease in PAI-1 activity and the increase in D-dimer levels (Figure 2).

Discussion

The main finding of this placebo-controlled double-blind study is that 6 weeks of treatment with oral estradiol and norethisterone significantly increased plasma levels of F1+2, soluble fibrin, and D-dimer, indicative of an increase in thrombin generation, fibrin generation and breakdown, and decreased PAI-1 activity, suggestive of a reduction in plasma fibrinolytic inhibitory activity. Furthermore, an association was evident between the increases in fibrin production and fibrinolysis but not between the changes in plasma fibrinolytic inhibitory activity and fibrinolysis.

F1+2 is a peptide generated as a result of the conversion of prothrombin to thrombin. Marked elevations of F1+2 occur with established venous thromboembolism23 and myocardial infarction,24 whereas lesser elevations have been reported in subjects at risk of these conditions,25,26 possibly heralding a prothrombotic state. Our finding of a 20% increase in F1+2 indicates that short-term treatment with estradiol and norethisterone increases in vivo thrombin generation. Elevated levels of F1+2 have also been reported with estrogen alone. An open crossover study showed a 25% increase after 6 weeks of treatment with 0.625 mg conjugated equine estrogen (CEE),14 and a randomized crossover study involving treatment for 3 months with CEE (either 0.625 or 1.25 mg) demonstrated a 40% and 98% increase in F1+2, respectively.15 On the other hand, results of placebo-controlled trials of combined estrogen and progestin therapy have been inconsistent. Six months of treatment with estradiol (2 mg/d) combined with cyclical micronized progesterone (200 mg/d from days 14 to 25) resulted in a 14% increase in F1+2 levels.20 However, 3 months of treatment with estradiol (1 or 1.5 mg) combined with cyclical nomegestrol acetate (2.5 mg, days 1 to 24)27 did not increase F1+2, whereas Walsh et al16 found a 16% increase in F1+2 after 3 to 6 months of treatment with CEE (0.625 mg) and continuous medroxyprogesterone acetate (2.5 mg/d), but this change was not statistically significant.

The formation of fibrin occurs via cleavage of fibrinogen, catalyzed by free thrombin. Yet free thrombin is subject to inhibitory control by protein binding.28 Thus, thrombin generation may occur without stimulating fibrin production, a situation previously defined as a prothrombotic state.24 Once the thrombin inhibitory capacity is exceeded, however, soluble fibrin is formed, with increased levels noted with venous thromboembolism29,30 and myocardial infarction.31 In the present study, we observed a 76% increase in soluble fibrin in
subjects given HRT, indicating that increased fibrin production accompanied the increased thrombin generation. Although effects of HRT on soluble fibrin have not been previously examined, estrogen alone has been demonstrated to cause a dose-dependent increase in fibrinopeptide A, a peptide also generated during fibrinogen cleavage, whereas the only study to examine the effect of combined HRT on fibrin production found no significant change in fibrinopeptide A levels.

Four factors potentially contributed to the variable findings in the literature regarding the effects of combined HRT on thrombin and fibrin formation. First, a variety of estrogens have been previously used. With the coagulation effects of conjugated estrogen demonstrated to be dose dependent, the variety of estrogens used may have had different potencies with regard to their effect on coagulation activity. Second, the reported studies have differed in the type and pattern (continuous or cyclic) of progestin administration, with potential implications because progestins may modify the actions of estrogen, may vary in many respects, including their androgenicity and metabolic effects, and may differ in their potential to cause venous thrombosis. Third, the duration of therapy varied between studies. We examined HRT use over 6 weeks, but other studies have had a duration of up to 6 months. Given evidence suggesting that hemostatic effects of HRT may be early and transient, these longer studies may have had a reduced power to detect any short-term but resolving changes in hemostatic markers. Last, study designs have varied, with single-center studies potentially advantaged by uniformity in blood collection and sample processing and larger multicenter studies having greater statistical power.

Under physiological circumstances, increases in fibrin formation may not result in thrombosis because of an accompanying increase in fibrinolysis. Fibrinolysis involves plasminogen conversion to plasmin via plasminogen activators, which are in turn inhibited by plasminogen activator inhibitors, including PAI-1. The role of PAI-1 as a fibrinolytic inhibitor appears clinically important, with elevations associated with venous thrombosis and myocardial infarction. In the present study, a 25% reduction in PAI-1 activity was observed after 6 weeks of combined HRT, consistent with other prospective studies using either estrogen alone or combined HRT and indicating that HRT with estradiol and norethisterone increases plasma fibrinolytic inhibitory activity.

The fibrinolytic process involves plasmin-mediated breakdown of cross-linked fibrin to produce D-dimer. D-dimer levels are elevated in venous thrombosis, pulmonary embolism, stroke, and myocardial infarction, with additional evidence that increased D-dimer levels may be a marker of subsequent thrombotic events. Our finding of significantly
The effects of HRT on coagulation and fibrinolysis are of clinical relevance in view of recent evidence suggesting that HRT is associated with an increased risk of arterial and venous thrombotic events. A 3-fold increase in venous thromboembolism with HRT use has been observed in case-control studies and in the recent Heart and Estrogen-Progestin Replacement Study (HERS), a double-blind, placebo-controlled, randomized study of combined oral HRT in the secondary prevention of cardiovascular disease. The HERS study also found an early increase in arterial vascular events associated with HRT use. It was hypothesized that this may be due to the prothrombotic effect of combined HRT, a suggestion consistent with a retrospective study noting increased unstable angina in women starting HRT after myocardial infarction compared with women not given HRT. Our finding that combined estradiol and norethisterone increases coagulation activation provides mechanistic evidence that this combination may also contribute to thrombosis in susceptible individuals.

In the present study, we have demonstrated that oral combined continuous HRT with estradiol and norethisterone results in the activation of coagulation, increased fibrinolysis, and reduced plasma fibrinolytic inhibitory activity. Increased fibrinolysis with HRT was positively associated with increased fibrin production but not with reduction in plasma fibrinolytic inhibitory activity. The finding of an increase in thrombin and fibrin generation is consistent with an adverse effect of combined HRT on thrombotic risk.

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References

Elevated D-dimer levels with HRT indicates that combined estradiol and norethisterone increases fibrinolysis. Two previous studies addressed the effects of combined HRT on fibrinolysis with divergent findings. Scarabin et al found no increase in D-dimer after 6 months of treatment with estradiol and micronized progesterone, whereas Koh et al recently reported increased fibrinolysis associated with decreased PAI-1 levels after 1 month of treatment with combined continuous HRT (0.625 mg of CEE and 2.5 mg medroxyprogesterone acetate), although the actual change in D-dimer levels in that study was not statistically significant.

We also aimed to investigate the effects of combined HRT on the relation between coagulation activation, plasma fibrinolytic inhibitory activity, and fibrinolysis. We have demonstrated a relation between fibrin production and fibrinolysis, with a positive correlation between baseline levels of soluble fibrin and D-dimer and also between the increases observed in these markers after HRT treatment. Alternatively, a relation between plasma fibrinolytic inhibitory activity and fibrinolysis was not found as there was no correlation evident between PAI-1 activity and D-dimer levels at baseline or after treatment. Although a contribution from enhanced plasma fibrinolytic inhibitory activity in the observed increase in fibrinolysis in the subjects receiving HRT was not excluded, an implication of these findings is that reactive fibrinolysis secondary to elevation in fibrin is a more important factor. In contrast, however, Koh et al found an inverse correlation between baseline D-dimer levels and PAI-1 antigen and between the increase in D-dimer and reduction in PAI-1 with HRT; the authors concluded that increased fibrinolysis was related to the enhanced plasma fibrinolytic inhibitory activity. On the other hand, Scarabin et al noted no increase in D-dimer despite the enhancement of plasma fibrinolytic inhibitory activity, evidenced by decreased levels of both PAI-1 antigen and activity and an increase in global fibrinolytic activity. Neither of these studies measured soluble fibrin levels; therefore, Koh et al were unable to address whether coagulation activation with a resultant increase in fibrin production was an alternative stimulus to fibrinolysis in their study, whereas Scarabin et al were unable to determine whether the absence of an increase in D-dimer, despite the observed elevation in F1 + 2 and thus thrombin generation, was related to effective antithrombin mechanisms and an absence of increase in fibrin in their study.

The mechanism of combined HRT effects on hemostatic markers was further evaluated by assessing the relation between changes in lipid and hemostatic parameters in the HRT-treated group on the basis of the well-established benefits of HRT on lipid profiles and the known association between lipids and hemostatic markers. However, although significant associations of baseline hemostatic marker levels with BMI and lipid subfractions were evident in the present study, no significant correlations between changes in lipids and hemostatic parameters were evident after treatment. This suggests that changes in hemostatic markers with HRT may be independent of changes in lipids. Only total cholesterol was lower in the HRT group after 6 weeks of treatment, whereas LDL and HDL remained unchanged. This was not unexpected, because on the basis of previous literature, the present study was underpowered to detect lipid changes.


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