Effects of combined oral hormone replacement therapy on tissue factor pathway inhibitor and factor VII

Roger E. PEVERILL*, Helena J. TEEDE†, Joseph J. SMOLICH*, Erica MALAN*, Dimitra KOTSOPOULOS†, Peter G. TIPPING‡ and Barry P. McGRATH†

*Centre for Heart and Chest Research, Department of Medicine, Monash Medical Centre and Monash University, 246 Clayton Road, Clayton, Victoria 3168, Australia, †Vascular Research Group, Department of Medicine, Monash Medical Centre and Monash University, 246 Clayton Road, Clayton, Victoria 3168, Australia, and ‡Centre for Inflammatory Diseases, Department of Medicine, Monash Medical Centre and Monash University, 246 Clayton Road, Clayton, Victoria 3168, Australia

ABSTRACT

Oral combined hormone replacement therapy (HRT) with oestradiol and norethisterone increases plasma levels of prothrombin fragment 1+2 (F1+2), indicating an increase in thrombin generation, but the mechanisms underlying this increase are uncertain. The aim of this randomized, placebo-controlled study was to determine whether an increase in factor VII, a factor that combines with tissue factor to activate the extrinsic pathway, or a decrease in tissue factor pathway inhibitor (TFPI), an inhibitor of extrinsic pathway activation, may contribute to increases in thrombin generation occurring with HRT. Healthy postmenopausal women aged 50–75 years received placebo (n = 19) or oral combined HRT (n = 18) and had blood collected for measurement of factor VII coagulation activity (VIIc), activated factor VII (VIIa) and TFPI at baseline and at 6 weeks. Baseline characteristics were similar in the two groups, including age, body mass index and cholesterol levels. As reported previously, HRT increased the F1+2 concentration by 20%. Placebo had no effect on VIIc, VIIa or TFPI, but 6 weeks of combined HRT decreased VIIc [from 1.11 ± 0.06 (mean ± S.E.M.) to 1.03 ± 0.06 i.u./ml; P < 0.03], VIIa [from 43.9; 10.8–198.3 (median; range) to 35.0; 6.3–66.8 m-units/ml; P < 0.03] and TFPI [from 81.3 ± 6.5 to 60.4 ± 5.5 ng/ml; P < 0.0001]. The decrease in TFPI with HRT was not correlated with the elevation in F1+2 levels. In conclusion, the increase in thrombin generation seen with HRT is not due to an effect on factor VII; in addition, while a contribution from the decrease in TFPI is possible, increased thrombin generation is not directly related to the decrease in TFPI.

INTRODUCTION

Epidemiological studies have suggested that treatment with hormone replacement therapy (HRT) is associated with a decreased risk of cardiovascular events [1,2]. However, the HERS trial, a randomized trial of HRT in women with established coronary heart disease, found an increased risk of cardiovascular events in the first year of therapy, albeit with a trend to a reduction in events during subsequent follow-up [3]. The HERS investigators proposed that the adverse early effect on cardiovascular events was due to a prothrombotic effect of HRT [3]. In keeping with this, there was also a 3-fold increased risk of venous thrombosis seen with the use of HRT in the HERS trial [3], an effect that is consistent with data from observational studies [4–6]. Further

Key words: coagulation, factor VII, hormone replacement therapy, oestrogen, progestin.

Abbreviations: AT, antithrombin; F1+2, fragment 1+2; HDL-C, high-density lipoprotein cholesterol; HRT, hormone replacement therapy; LDL-C, low-density lipoprotein cholesterol; TAT, thrombin–antithrombin complex; TFPI, tissue factor pathway inhibitor; VIIa, activated factor VII; VIIc, factor VII coagulation activity.

Correspondence: Dr R. E. Peverill (e-mail roger.peverill@med.monash.edu.au).
support for a prothrombotic effect of HRT comes from a recent report that women on HRT with atrial fibrillation have an increased risk of thromboembolism [7].

Oestrogen appears to be a likely mediator of the prothrombotic effect of HRT, as it has been shown to cause a dose-dependent increase in plasma levels of prothrombin fragment 1+2 (F1+2), indicating an increase in thrombin production in vivo [8]. While the addition of a progestin to oestrogen in combined HRT may modify the effects of oestrogen [9], we have recently demonstrated that 6 weeks of HRT with the combination of oestradiol and norethisterone also increases plasma levels of F1+2 [10]. Although the mechanisms underlying this increase in thrombin generation are yet to be defined, several lines of evidence point to a role for the extrinsic coagulation pathway. Thus, initiation of the extrinsic pathway is mediated via binding of activated factor VII (VIIa) and tissue factor [11], and oestrogen has been reported to increase factor VII levels [12–17]. Furthermore, there is evidence for an effect of sex steroids on tissue factor pathway inhibitor (TFPI), the main physiological inhibitor of the tissue factor–VIIa complex [18], with TFPI levels being reduced in patients on the oral contraceptive pill [19].

The aim of the present double-blind, randomized, placebo-controlled study, performed in healthy postmenopausal women, was to determine the short-term effects of oral combined HRT with oestradiol and norethisterone on components of the coagulation pathway that could influence thrombin generation. Factor VII was assessed by measurement of VIIa and factor VII coagulation activity (VIIc), and TFPI was assessed by measurement of antigen levels. In addition, antithrombin (AT) was measured, as it is not only an important inhibitor of thrombin, but also an inhibitor of factors Xa and IXa, which precede thrombin in the coagulation cascade [11]. The thrombin–AT complex (TAT) was measured, as studies suggest that there may be divergent effects of HRT on thrombin generation and its inactivation through AT binding [14,17]. Lastly, the relationships between coagulation factors and lipids before and after treatment with HRT were also evaluated, since previous population studies have reported associations of plasma lipid levels with factor VII [20–22] and TFPI [23,24].

**METHODS**

**Subjects**

Subjects and study design have been described previously in detail [10]. A total of 42 healthy postmenopausal women were enrolled in a double-blind, placebo-controlled, randomized trial conducted over 6 weeks. Participants were aged 50–75 years, had not received HRT for at least 12 months, and had not had a hysterectomy or oophorectomy. Postmenopausal status was defined as the absence of menses for at least 12 months, a follicle-stimulating hormone level of > 20 i.u./l and an oestradiol level of < 120 pmol/l. 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 mmHg), a history of venous thrombosis, breast or endometrial cancer, abnormal uterine bleeding, abnormal cervical smear or mammogram results, or the presence of a major illness. After randomization using computer-generated random numbers, 22 women were assigned to HRT and 20 to placebo. Women commenced either continuous combined therapy with oral oestradiol (2 mg) combined with continuous norethisterone acetate (1 mg) (Kliogest; Novo-Nordisk) or placebo tablets having an identical appearance. Three subjects withdrew from the active therapy group, two because of vaginal bleeding and one because of unforeseen overseas travel. Ethical approval was obtained from the Monash Medical Centre Human Research and Ethics Committee, and all participants gave written informed consent prior to their enrolment in the study.

**Blood collection**

Gonadotrophins and serum oestradiol were measured at baseline. Fasting morning blood samples were collected at baseline and at 6 weeks via non-traumatic phlebotomy by a single technician for measurement of lipid profiles and haemostatic factors. Venepuncture was performed with a 19-gauge needle directly into plain tubes for lipid and hormone assays, then into two 3.8% citrate tubes (9:1 ratio) for coagulation studies. After immediate centrifugation of samples at 2500 g for 12 min, plasma was separated into 200 µl aliquots and stored at −80 °C; samples were thawed immediately prior to analysis.

**Lipoprotein assays**

Total cholesterol and triacylglycerols were measured using enzymic reagents (DADE Diagnostics, Brisbane, Australia). High-density lipoprotein cholesterol (HDL-C) was measured by homogeneous HDL-C assay techniques (HDL-C-Plus; DADE Diagnostics) adapted to a DADE Dimension RXL chemistry analyser (DADE Diagnostics). Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation [LDL-C = (total cholesterol − HDL-C) − (triacylglycerols×0.20)], adapted to S.I. units.

**Haemostatic factors**

All haemostatic markers were assayed by a single trained medical scientist, 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; Behringwerke AG, Marburg, Germany) and TAT (Enzygnost TAT; Behringwerke AG). AT was measured using a chromogenic assay kit (Instrumentation Laboratory, Milan, Italy). VIIc was measured in a one-stage clotting assay with factor VII-deficient plasma (Helena Laboratories, Melbourne, Australia) and Innovin thromboplastin (Dade/Behring, Coorparoo, Australia). VIIa was assayed by a clot-based technique utilizing a recombinant truncated tissue factor that allows only VIIa and not factor VII to activate coagulation (Staclot, VIIa-rTF; Diagnostica Stago, Asnieres-Sur-Seine, France). TFPI antigen was assayed by an ELISA that detects intact and truncated forms of TFPI, as well as TFPI complexed with tissue factor and VIIa (Imubind Total TFPI ELISA; American Diagnostica, Greenwich, CT, U.S.A.).

Statistical analysis
Statistical calculations were performed using SPSS version 8 (SPSS Inc., Chicago, IL, U.S.A.). Data were normally distributed, except for VIIa, which has been expressed as median (range). Other parameters are expressed as means ± S.E.M. Baseline measurements were compared using an unpaired t test, or a non-parametric test if not normally distributed. Baseline and 6-week measurements were compared with a paired t test. Baseline and 6-week measurements of VIIa were also compared with a paired t test after logarithmic transformation of the data. Univariate analyses were performed using Pearson’s (parametric data) or Spearman’s (non-parametric data) correlation coefficients. Significance was accepted at the P < 0.05 level. Paired blood samples were available for 20 placebo patients and 19 active therapy patients, but one patient from each group was excluded from the analysis because of baseline soluble fibrin levels or F1+2 levels greater than 10 S.D.s above the mean.

RESULTS
Univariate analysis of the relationships between haemostatic parameters and cardiovascular risk factors at baseline in the cohort of 37 patients demonstrated positive correlations between VIIc and VIIa (r = 0.74, P < 0.0001; Figure 1), TFPI and total cholesterol (r = 0.53, P < 0.001), and TFPI and LDL-C (r = 0.50, P < 0.001; Figure 2). There was a trend to a positive correlation between TFPI and age (r = 0.29, P = 0.065). F1+2 was not related to VIIc, VIIa, TFPI, TAT or AT. Moreover, no correlations were evident between any of the haemostatic factors and HDL-C, triacylglycerols or body mass index.

Baseline and 6-week lipid profiles and F1+2 levels in the study group have been reported previously [10]. In the HRT group, there was a 12% decrease in total cholesterol and an 11% decrease in LDL-C, although the latter change was not statistically significant. HRT use also resulted in an ~20% increase in the level of F1+2 [10]. Table 1 shows the mean levels of haemostatic factors/markers at baseline and after 6 weeks of oestradiol and norethisterone. There were no differences in baseline levels between the placebo and HRT groups, and there were no significant changes in any of the variables between baseline and 6 weeks in the placebo group. In the HRT group, there were decreases in the levels of VIIc and VIIa (by ~7% and ~20% respectively), and the changes in these levels were positively correlated (r = 0.61, P = 0.006; Figure 3). The level of TFPI also decreased in the HRT group (by ~26%), and there was a trend to a positive correlation between the changes in TFPI and LDL-C (r = 0.44, P = 0.066). No relationship was evident between the decrease in TFPI and the increase in F1+2.
Table 1  Haemostatic factors at baseline and at 6 weeks, according to treatment group
Results are expressed as means ± S.E.M., except those for VIIa, which are expressed as median (range). Listed P values are for differences between groups. Significance of differences compared with values at baseline are indicated by: * P<0.03, ** P<0.02, *** P<0.0001.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Placebo (n = 19)</th>
<th>HRT (n = 18)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIIc (i.u./ml)</td>
<td>Baseline</td>
<td>1.17 ± 0.04</td>
<td>1.11 ± 0.06</td>
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<tr>
<td></td>
<td>6 weeks</td>
<td>1.17 ± 0.04</td>
<td>1.03 ± 0.06*</td>
</tr>
<tr>
<td>VIl a (m-units/ml)</td>
<td>Baseline</td>
<td>52.5 (21.4–101.5)</td>
<td>43.9 (10.8–198.3)</td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td>55.0 (20.5–94.9)</td>
<td>35.0 (6.3–66.8)*</td>
</tr>
<tr>
<td>TFPI antigen (ng/ml)</td>
<td>Baseline</td>
<td>79.8 ± 3.1</td>
<td>81.3 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td>81.4 ± 2.8</td>
<td>60.4 ± 5.5***</td>
</tr>
<tr>
<td>AT (i.u./ml)</td>
<td>Baseline</td>
<td>1.06 ± 0.02</td>
<td>1.08 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td>1.06 ± 0.02</td>
<td>1.05 ± 0.02**</td>
</tr>
<tr>
<td>TAT (µg/l)</td>
<td>Baseline</td>
<td>1.71 ± 0.30</td>
<td>3.27 ± 0.82</td>
</tr>
<tr>
<td></td>
<td>6 weeks</td>
<td>2.03 ± 0.37</td>
<td>4.71 ± 1.25</td>
</tr>
</tbody>
</table>

Figure 3  Scatterplot of the relationship between changes in VIIc and VIIa in patients receiving combined HRT

The effects of HRT on AT and TAT were less clear, because, while there was a small but significant decrease in AT in the HRT group, no difference was evident in the 6-week levels of AT between the placebo and HRT groups (Table 1). Further, while the level of TAT in the HRT group exceeded the level in the placebo group at 6 weeks, the change in TAT in the HRT group was not statistically significant (P = 0.15; Table 1). On linear regression analysis in patients receiving HRT, there was no significant correlation between the changes in TAT and F1+2.

DISCUSSION

This randomized, placebo-controlled clinical trial has found a significant effect of 6 weeks of treatment with oestradiol and norethisterone on factors responsible for the initiation and inhibition of the extrinsic coagulation pathway. HRT use was associated with decreases in VIIa and VIIc, suggesting a decreased tendency for extrinsic pathway activation, and also with a reduction in TFPI antigen concentration, indicating a reduced potential to inhibit extrinsic pathway activation. These findings suggest that increases in thrombin generation with oestradiol and norethisterone occur in spite of, rather than as a result of, changes in factor VII activation. Furthermore, while a contribution of a reduction in TFPI could provide a mechanism for the increase in thrombin generation in vivo with this HRT combination, there was no relationship between the decrease in TFPI and the increase in F1+2.

This is the first study to determine the effects of oral combined HRT on TFPI. We found that 6 weeks of oestradiol and norethisterone resulted in a 26% decrease in TFPI antigen levels. This reduction is of a similar degree to the 25% decrease in the level of TFPI antigen reported previously in women on the oral contraceptive pill [19]. Although TFPI activity was not measured in the present study, oral contraceptive pill use is associated with lower levels of both TFPI antigen and activity [19], suggesting that a reduction in TFPI antigen with sex steroids is associated with a reduction in TFPI activity.

TFPI is the main inhibitor of the extrinsic pathway of coagulation. It inhibits factor Xa in a calcium-independent manner and binds VIIa–tissue factor in the presence of calcium [11]. A decrease in TFPI could therefore reduce the inhibition of the extrinsic pathway, resulting in downstream activation of coagulation and an increase in thrombin generation. However, while not excluding an effect of TFPI, a direct relationship between the decrease in TFPI antigen and the increase in thrombin generation was not supported by our study, as there was no correlation between the decrease in TFPI antigen and the increase in F1+2 level.

A significant proportion of TFPI in plasma is associated with lipoproteins, and TFPI activity is higher...
in patients with hyperlipidaemia [23]. Consistent with previous reports, we found that TFPI antigen was positively correlated with the concentration of LDL-C [23]. Furthermore, there was a trend to a positive correlation between the decreases in LDL-C and TFPI in the patient group receiving HRT, suggesting that the decrease in TFPI may have been due, in part, to the effect of HRT in reducing LDL-C. However, given that there was a 26% decrease in TFPI, the change in lipoprotein levels with HRT at most only accounts for a small proportion of the reduction in TFPI activity. Moreover, the possibility that sex steroids can influence TFPI by a mechanism other than via lipids was suggested in the study of Harris et al. [19], where lower levels of TFPI in patients taking the oral contraceptive pill were not associated with a lower LDL-C level.

The tissue-factor–VIIa complex is an important stimulator of the extrinsic pathway of the coagulation cascade [11], and changes in VIIa could thus influence the thrombin generation that occurs in vivo due to oestradiol and norethisterone. However, 6 weeks of treatment with oestradiol and norethisterone actually resulted in a reduction in VIIa, indicating that changes in VIIa are more likely to have a restraining influence on the increase in thrombin generation. Interestingly, this change is opposite to that which was observed with the oral contraceptive pill, the use of which was associated with a higher level of VIIa [19]. Our finding also differs from other studies with combined HRT using different oestrogen/progestin combinations and different durations of therapy. Scarabin et al. [25] found no change in VIIa in postmenopausal women after 6 months of therapy with oestradiol and micronized progesterone, and van Baal et al. [17] reported no change in VIIa after 12 weeks of micronized oestradiol with a progestin. Importantly, as both studies also reported increased levels of F1 + 2, they provide additional evidence that changes in thrombin generation with combined HRT occur independently of VIIa [17,25].

The decrease in VIIa with HRT observed in the present study could have been due to a decrease in the concentration of the substrate (i.e. factor VII antigen), a decrease in the degree of activation of factor VII to VIIa, or a combination of these mechanisms. Although factor VII antigen was not measured, it is likely that a decrease in this antigen played a role in the decrease in VIIa, because a reduction in factor VII antigen has been reported previously with the same dose of oestradiol and norethisterone as used in the present study [26]. However, whether a decrease in the degree of activation of VII also contributed to the decrease in VIIa cannot be deduced without a comparison of the effects of HRT on the VIIa/factor VII antigen ratio.

In our study, HRT caused a decrease in VIIc, which was correlated with the fall in VIIa. This relationship between changes in VIIa and VIIc was expected, since, while VIIc is measured by the conversion of factor VII into VIIa, it is also dependent on preformed VIIa [27]. Previous studies have demonstrated considerable heterogeneity in the effects of HRT on VIIc, although these apparent differences may be due largely to dose-dependent effects of progestins. Thus most studies using oral oestradiol alone have reported an increase in VIIc [12–17], whereas a number of studies using combined HRT found no change [15,16,25,28], suggesting that progestins may suppress the effect of oestradiol on VIIc. Furthermore, studies of combined HRT using oestradiol and norethisterone have reported a similar reduction in VIIc to that observed in the present study. Sporrong et al. [26] reported a decrease in VIIc with 2 mg of oestradiol and 1 mg of norethisterone after 1, 4 and 12 months of treatment, and Samsioe et al. [29] found a decrease in VIIc with low-dose oestradiol (1 mg) and norethisterone (0.5 mg or 0.25 mg) after 3, 6 and 12 months of treatment. That the decrease in VIIc in the latter study was a dose-dependent effect of norethisterone was suggested by the finding that there was a greater reduction in VIIc in the group receiving the higher dose of norethisterone.

AT is an important inhibitor of factors IXa and Xa, as well as an inhibitor of thrombin activity [11], and thus a reduction in AT levels could potentially contribute to an enhancement of coagulation processes. Oral oestrogen therapy has resulted in decreased levels of AT in most [8,12,14,15,17], but not all [16], studies. On the other hand, the results of previous studies with combined HRT have been more variable, with reports of AT being decreased [15,25,26,29] or unchanged [16,17,30,31]. We found a small but significant decrease in AT (~3%) with oestradiol and norethisterone, although there was no significant difference between the placebo and HRT groups in the levels of AT at 6 weeks. Two other studies using oestradiol and norethisterone reported a decrease in AT of between 5 and 10% [26,29]. Whether the decrease in AT is due to reduced production, and thus has the potential to increase both thrombin generation and activity, or, alternatively, whether it might be secondary to increased thrombin generation, and thus an increase in AT binding and clearance, is unknown.

TAT is formed when thrombin binds with AT, and increases in TAT normally parallel increases in F1 + 2 [32]. However, in one previous study of HRT, conjugated equine oestradiol resulted in an increase in F1 + 2, but a decrease in TAT [14]. A possible explanation for this finding is that it may have been due to a decrease in available AT, occurring independently of the increase in thrombin generation. Consistent with this, van Baal et al. [17] found that oestradiol use resulted in an increase in F1 + 2, a reduction in AT, but no change in TAT. On the other hand, we found in the present study that the level of TAT in the HRT group exceeded the level in the placebo group after 6 weeks of HRT, although the change in TAT in the HRT group was not statistically significant.
effects of combined HRT on TAT remain unresolved, although our data make it unlikely that treatment with oestradiol and norethisterone causes a decrease in TAT.

A limitation of our study, similar to most studies investigating the effects of HRT on coagulation, was the small number of subjects. Therefore we cannot exclude the possibility that the lack of significant changes in AT and TAT with HRT was due to a type II error. However, our finding that increases in thrombin generation occurred despite decreases in VIIc and VIIa is unlikely to be related to sample size, as the reductions in VIIc and VIIa were statistically significant. The lack of a correlation between the reduction in TFPI and the increase in F1 + 2 could be a type II error, and does not exclude the possibility that the decrease in TFPI may affect thrombin generation.

In conclusion, this study in postmenopausal women examined the effects of 6 weeks of oral combined HRT with oestradiol and norethisterone on factors responsible for the initiation and inhibition of the extrinsic pathway of the coagulation cascade, and found that HRT decreased VIIc, VIIa and TFPI functional activity. The increase in thrombin generation reported previously with the use of oestradiol and norethisterone [10] therefore occurs in spite of a decrease in VIIa. Furthermore, although a reduction in TFPI may be a contributory factor to an increase in thrombin generation, no relationship was apparent between the changes in thrombin generation and TFPI. These findings suggest that there may be other, as yet unidentified, factors that are primarily responsible for the increase in thrombin generation seen with oestradiol and norethisterone treatment.

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