Vascular Effects of Synthetic or Natural Progestagen Combined With Conjugated Equine Estrogen in Healthy Postmenopausal Women

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Background—Synthetic, not natural, progestagen may negate the favorable effects of estrogen. Nonetheless, observational studies report no differences in risk for clinical cardiovascular events between users of unopposed estrogen and users of estrogen combined with synthetic progestin.

Methods and Results—In a double-blind study, we randomly assigned 20 healthy postmenopausal women to micronized progesterone (MP) 200 mg or medroxyprogesterone acetate (MPA) 10 mg for 10 days with conjugated equine estrogen (CEE) 0.625 mg for 25 days and the remaining 5 days off cyclically during 2 months, followed by crossover to the alternate therapy. CEE+MP and CEE+MPA significantly improved the percent flow-mediated dilator response to hyperemia relative to baseline measurements (P=0.004 by ANOVA) by a similar degree (P=0.863). Both therapies significantly decreased E-selectin, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 levels from baseline values (P<0.001, P=0.048, and P=0.016 by ANOVA, respectively) by a similar degree (P=0.977 for ICAM-1 and P=0.541 for VCAM-1, respectively). CEE+MPA decreased E-selectin levels more than CEE+MP did (P=0.040). Both therapies significantly decreased monocyte chemoattractant protein-1 levels from baseline values (P<0.005 by ANOVA) by a similar degree (P=0.194). Both therapies significantly decreased tissue factor antigen and increased tissue factor activity levels from baseline values (P=0.003 and P<0.001 by ANOVA, respectively) by a similar degree (P=0.652 for antigen and P=0.173 for activity). Both therapies significantly lowered plasma plasminogen activator inhibitor-1 levels from baseline values (P<0.001 by ANOVA) by a similar degree (P=0.533).

Conclusions—CEE+MP and CEE+MPA provide similar improvement in endothelium-dependent vasodilator responsiveness and effects on markers of inflammation, hemostasis, and fibrinolysis inhibition in healthy postmenopausal women. (Circulation. 2001;103:1961-1966.)

Key Words: atherosclerosis ■ endothelium ■ proteins ■ cell adhesion molecules ■ fibrinolysis

Prospective cohort surveys suggest that estrogen therapy decreases the risk of coronary artery disease in postmenopausal women initially healthy at time of enrollment. The mechanisms of this apparent benefit of hormone therapy probably include lipoprotein effects. Other mechanisms of potential benefit include improvement in endothelium-dependent vasodilator function due to increased nitric oxide (NO) bioavailability and potentiation of fibrinolysis.

The nuclear transcription factor NF-κB activates transcription of genes encoding adhesion molecules and chemoattractant factors, such as monocyte chemotactic peptide, that attract monocytes into the vessel wall, converting them into macrophages with the potential release of reactive oxygen species and prothrombogenic peptides, such as tissue factor. Recent studies indicate that NO may protect NF-κB from activation by oxidized LDL or cytokines and thus prevent or attenuate the transcription and expression of adhesion molecules, monocyte chemoattractant protein (MCP)-1, and possibly tissue factor on the endothelial cell surface.

Therapies that increase NO bioactivity may reduce synthesis of proinflammatory proteins and MCP-1 on the endothelial cell surface. In this regard, we have previously shown that unopposed conjugated equine estrogen (CEE) improved endothelium-dependent vasodilator responsiveness, an index of NO bioactivity and reduced markers of inflammation. Studies suggest, however, that synthetic, not natural, progestagen may negate the favorable effects of estrogen. Nonetheless, observational studies of hormone replacement ther-

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therapy report no differences in risk for clinical cardiovascular events between users of unopposed estrogen and users of estrogen combined with progestins, including women using predominantly medroxyprogesterone acetate (MPA).1,11,12 Vascular inflammation plays an important role in the pathogenesis of atherosclerosis and may contribute to increasing the risk of myocardial infarction.13,14 Accordingly, therapies that reduce vascular inflammation may reduce cardiovascular risk. Although inflammation of the arterial wall in the vicinity of atherosclerotic plaques is commonly found at necropsy, this manner of demonstration is hardly useful for identifying patients at risk for myocardial infarction. Impaired fibrinolysis as measured by an elevation in plasminogen activator inhibitor type 1 (PAI-1) is predictive of ischemic heart disease.1,15 Accordingly, such markers as cell adhesion molecules, MCP-1, tissue factor, and PAI-1 measured in plasma may be useful surrogates for vascular inflammation and thrombus formation. Thus, we investigated the effects of synthetic or natural progestagen combined with CEE on vasomotor function and serological markers of homeostatic functions potentially affected by NO-potentiating properties in healthy postmenopausal women.

Methods

Study Population and Design

Twenty healthy postmenopausal women (55±8 years old, mean±SD) participated in this study, all with plasma 17β-estradiol levels <50 pg/mL and cessation of menses for ≥1 year. Baseline total cholesterol, triglyceride, HDL cholesterol, and LDL cholesterol levels were 213±38, 132±53, 58±10, and 129±35 mg/dL, respectively. None were diabetic, hypertensive, or a current cigarette smoker. No subject had taken any medications during the preceding 2 months. This study was randomized, double-blind, crossover in design. Study participants received micronized progesterone (MP) 200 mg or MPA for 10 days (from days 16 to 25) with CEE 0.625 mg for the first 25 days and the remaining 5 days off cyclically during 2 months, with the second treatment period initiated on completion of the first treatment period. The study was approved by the Gil Hospital Institute Review Board, and all participants gave written, informed consent.

Laboratory Assays

Blood samples for laboratory assays and vascular studies were obtained at approximately 8:00 AM after overnight fasting except for hormone replacement, at baseline and at the second 23rd to 25th day of each treatment period and were immediately coded so that investigators performing laboratory assays were blinded to subject identity or study sequence. Assays for lipids, E-selectin, intercellular adhesion molecule type 1 (ICAM-1), vascular cell adhesion molecule type 1 (VCAM-1), and PAI-1 antigen were performed as previously described.8,9 MCP-1 levels were measured in duplicate by ELISA (R & D Systems). Tissue factor antigen and activity were measured in duplicate by ELISA and Actichrome assays (American Diagnostica). The interassay and intra-assay coefficients of variation were <8%.

Vascular Studies

Imaging studies of the right brachial artery were performed with an ATL HDI 3000 ultrasound machine equipped with a 10-MHz linear-array transducer on the basis of a previously published technique.8,9,16 All images were transmitted to a personal computer via Ethernet with DICOM format (Digital Imaging and Communication in Medicine) and then saved on the hard disk of a personal computer as a BMP format. Arterial diameters were measured with Image Tool for Windows version 2.0 (University of Texas Health Science Center, San Antonio). Measurements were performed by 2 independent radiologists (S.K.L. and H.Y.H.) blinded to the subject’s identity or study sequence. Assays for lipids, E-selectin, intercellular adhesion molecule type 1 (ICAM-1), vascular cell adhesion molecule type 1 (VCAM-1), and PAI-1 antigen were performed as previously described.8,9 MCP-1 levels were measured in duplicate by ELISA (R & D Systems). Tissue factor antigen and activity were measured in duplicate by ELISA and Actichrome assays (American Diagnostica). The interassay and intra-assay coefficients of variation were <8%.

Statistical Analysis

Data are expressed as mean±SD After testing data for normality, we compared values at baseline and after each therapy, as reported in the Table. We presumed that the second baseline after the washout was not different from the first baseline, because we determined no carryover effect of CEE and progestagen for 6 weeks from our previous studies.4,5,9 and thus, we decided on 2 months as the treatment period without washout and the second baseline. Indeed, we found no carryover effect in this study (see Results). The effects of the 2 therapies on vascular function and markers of inflammation,

| Table. Effects of Oral CEE Combined With MP or MPA on Endothelial Function |
|----------------|----------------|----------------|
|                | Baseline       | CEE+MP         | CEE+MP         |
| Vasomotor function |                |                |                |
| Flow-mediated dilation, % | 4.62±2.66      | 7.41±3.46†      | 7.54±4.45†      |
| Nitroglycerin dilation, % | 13.25±3.84     | 11.42±4.76     | 13.54±4.73     |
| Cell adhesion molecules |                |                |                |
| E-selectin, ng/mL | 41.7±15.0      | 35.9±13.1†      | 32.0±11.7†      |
| ICAM-1, ng/mL    | 358±143        | 313±135        | 308±114*       |
| VCAM-1, ng/mL    | 846±278        | 661±209†       | 672±182†       |
| MCP-1, pg/mL     | 146±44         | 124±37†        | 130±40†        |
| Hemostasis       |                |                |                |
| Tissue factor antigen, pg/mL | 223±42       | 194±38†        | 189±42†        |
| Tissue factor activity, nmol/L | 0.854±0.258   | 1.455±0.584‡   | 1.275±0.432‡   |
| Fibrinolysis     |                |                |                |
| PAI-1, ng/mL     | 33.02±12.43    | 22.78±8.36‡    | 23.73±10.68†   |

Data are expressed as mean±SD.

*P<0.05; †P<0.01; ‡P<0.001; §P=0.069 vs baseline. |P<0.05 vs CEE+MP.
hemostasis, and fibrinolysis inhibition relative to baseline values were analyzed by 1-way repeated-measures ANOVA or Friedman’s repeated ANOVA on ranks. After demonstration of significant differences among therapies by ANOVA, post hoc comparisons between treatment pairs were made by use of the Student-Newman-Keuls multiple comparison procedures. Pearson correlation coefficient analysis was used to assess associations between measured parameters. We calculated that 20 subjects would provide 80% power for detecting difference of absolute increase, $\alpha = 0.05$ on the basis of our previous studies $8,9$ and others.$^{17}$ The comparison of endothelium-dependent dilation between the 2 treatment schemes was prospectively designated as the primary end point. All other comparisons were considered secondary. Therefore, probability values less than the Bonferroni-adjusted $\alpha$ of 0.05/7 = 0.007 were deemed statistically significant for the secondary end points.

**Results**

To assess the possibility of a carryover effect from the initial treatment periods to the next treatment period, we compared the percent changes of (1) the first treatment CEE+MP and the first treatment CEE+MPA after 2 months, (2) the cumulative effect of both therapies after 4 months, (3) the first treatment CEE+MP and the second treatment CEE+MP, and (4) the first treatment CEE+MPA and the second treatment CEE+MPA, relative to baseline values. There were no significant differences in age and baseline values—vascular function (diameter and flow) and markers of inflammation, hemostasis, and fibrinolysis inhibition—between each group. No significant differences were found in the above 4 comparisons (data not shown).

**Effects of Therapies on Vasomotor Function**

Basal brachial artery diameter and forearm blood flows were similar during the 2 treatment periods, as were the peak brachial artery diameters and forearm blood flows during reactive hyperemia and the percent increase in flow during hyperemia. Both therapies significantly improved the percent flow-mediated dilator response to hyperemia relative to baseline measurements ($P=0.004$ by ANOVA; Figure 1) by a similar degree ($P=0.863$). The brachial artery dilator response to nitroglycerin between each therapy was not significantly changed from baseline measurements ($P=0.274$ by ANOVA; Table).

**Effects of Therapies on Markers of Inflammation**

Both therapies significantly decreased E-selectin, ICAM-1, and VCAM-1 levels from baseline values ($P<0.001$, $P=0.048$, and $P=0.016$ by ANOVA, respectively; Table). CEE+MPA decreased E-selectin levels more than CEE+MP did ($P=0.040$). CEE+MPA significantly decreased ICAM-1 levels by 8 ± 33% from baseline values, although this effect was not significantly greater than the weakly significant 8 ± 33% reduction on CEE+MP ($P=0.977$). In VCAM-1, there were no significant differences between each therapy ($P=0.541$).

Both therapies significantly decreased MCP-1 levels from baseline values ($P<0.005$ by ANOVA; Table and Figure 2). CEE+MPA tended to decrease MCP-1 levels by 7 ± 30% from baseline values, although this effect was not significantly greater than the significant 13 ± 18% reduction with CEE+MP ($P=0.194$).

There was a statistically significant correlation between the reduction in E-selectin levels and increase in flow-mediated dilation on CEE+MP ($r=--0.552$, $P=0.041$) but not CEE+MPA ($r=--0.064$, $P=0.835$). Otherwise, there was no association between changes in ICAM-1, VCAM-1, or MCP-1 levels and changes in flow-mediated dilation of the brachial artery (all $r>=0.286$). There were strong associations between E-selectin, ICAM-1, and VCAM-1 levels. There were statistically significant correlations between the changes in E-selectin levels and changes in ICAM-1 levels on CEE+MP ($r=0.575$, $P=0.005$) and also CEE+MPA ($r=0.686$, $P=0.0004$), between the changes in E-selectin levels and changes in VCAM-1 levels on CEE+MP ($r=0.609$, $P=0.003$) and also CEE+MPA ($r=0.538$, $P=0.010$), and between the changes in ICAM-1 levels and changes in VCAM-1 levels on CEE+MP ($r=0.735$, $P=0.0001$) and also CEE+MPA ($r=0.669$, $P=0.0007$).
There was no association, however, between changes in E-selectin, ICAM-1, and VCAM-1 levels and changes in MCP-1 levels (all $r<0.024$).

**Effects of Therapies on Hemostasis and Fibrinolysis Inhibition**

Both therapies significantly decreased tissue factor antigen and increased tissue factor activity levels from baseline values ($P=0.003$ and $P<0.001$ by ANOVA, respectively; Table and Figure 3) by a similar degree ($P=0.652$ for antigen and $P=0.173$ for activity). Both therapies significantly lowered plasma PAI-1 levels from baseline values ($P<0.001$ by ANOVA; Table and Figure 4) by a similar degree ($P=0.533$). There was no association, however, between percent changes in tissue factor antigen or tissue factor activity and percent changes in PAI-1 antigen (all $r<0.268$).

There was no significant correlation between changes in tissue factor antigen levels and increase in flow-mediated dilation on CEE+MP ($r=0.183$) or CEE+MPA ($r=-0.028$). Similarly, there was no significant correlation between changes in tissue factor antigen levels and changes in MCP-1 levels on CEE+MP ($r=0.145$) or CEE+MPA ($r=-0.074$).

**Discussion**

Estrogen combined with progestagen has been recommended in postmenopausal women having a uterus to prevent endometrial hyperplasia or malignancy. We investigated the effects of synthetic and natural progestagens on endothelium, because endothelial dysfunction may contribute to development and clinical expression of atherosclerosis, including coronary heart disease.

Estrogen has been shown in endothelial cell culture studies to increase transcription and activity of NO synthase. Gerhard et al. observed that intravaginal micronized progesterone added to estradiol therapy did not significantly attenuate the improvement in flow-mediated dilation that was observed with estradiol administered alone in 17 postmenopausal women with mild hypercholesterolemia. Herrington et al. reported that MPA 2.5 mg combined with CEE 0.625 mg daily significantly improved flow-mediated dilation of the brachial artery in postmenopausal women with coronary artery disease. In contrast, Sorensen and coworkers reported that cyclical estradiol and norethisterone administered for 2.9 years did not improve endothelial function, measured as brachial artery flow-mediated vasodilation. We observed that synthetic progesterin improved flow-mediated brachial artery dilator response to hyperemia comparable to natural progesterone in the present study. Given the standard deviation, 3.22%, of differences of flow-mediated dilation between CEE+MP and CEE+MPA, the statistical power to accept our observation was 83%. Furthermore, the magnitude of improvement was relatively similar to our previous studies with unopposed estrogen and others. Indeed, CEE alone improved brachial artery flow-mediated vasodilation from 4.3% to 8.8%.

To gain additional insight as to mechanisms of potential vasculoprotective effects of hormone replacement therapy, we measured markers of inflammation, hemostasis, and fibrinolysis inhibition that clinical and experimental studies indicate are potentially affected by these therapies.

Experimental evidence suggests that cell adhesion molecules, once expressed on the endothelial cell surface, may be shed from the surface. Several groups have reported the presence of E-selectin, ICAM-1, and VCAM-1 in the culture supernatant within 4 to 6 hours of endothelial or leukocyte...
cell activation and in sera of humans, as shown by the same monoclonal antibody assay as used to demonstrate adhesion molecules in the supernatant of activated endothelial cells in culture. Although the biological function in sera remains unclear, the clinical relevance of cell adhesion molecules has been suggested by several observational studies. In the Atherosclerosis Risk in Communities study, higher serum levels of E-selectin and ICAM-1 were found in patients with coronary heart disease and carotid artery atherosclerosis than in healthy control subjects. E-selectin levels correlated positively with the carotid artery thickness measured by ultrasound in this study. As to its clinical significance, men in the Physician’s Health Study in the highest quartile of ICAM-1 levels were found to be at greater cardiovascular risk than men in the lowest quartile. Plasma concentrations of ICAM-1 increased with increasing prevalence of the usual cardiovascular risk factors in healthy men. A recent study demonstrated that serum concentrations of E-selectin, ICAM-1, and VCAM-1 have been reported to be higher in postmenopausal women with coronary artery disease not on hormone therapy than in postmenopausal women with coronary artery disease on hormone therapy. We observed that both therapies significantly decreased E-selectin, ICAM-1, and VCAM-1 levels from baseline values, as in our previous studies showing 13%, 7%, and 6% reduction with CEE alone. Furthermore, there were no significant differences between each therapy except for E-selectin. The apparently greater effect of synthetic progestin than natural progesterone on E-selectin levels was small and of marginal statistical significance, however, and therefore may not be biologically significant.

Reckless et al demonstrated that elevated expression of MCP-1 was correlated with vascular macrophage accumulation in apolipoprotein(a)-transgenic mice. Frazier-Jessen et al observed that estradiol inhibited LPS-stimulated JE/MCP-1 mRNA expression in ANA-1 and J774A.1 murine macrophage cell lines. Pervin et al observed that the cholesterol-induced increase in MCP-1 protein and mRNA expression was significantly attenuated in ovariectomized rabbits with physiological concentrations of estradiol. We found that both therapies significantly decreased MCP-1 levels from baseline values, consistent with experimental reports. Although the biological function in sera remains unclear, one recent study demonstrated that plasma MCP-1 levels were elevated in patients with acute coronary syndrome and that enalapril therapy significantly reduced plasma MCP-1 levels compared with placebo.

Tissue factor serves as a cofactor for plasma factor VII and a cellular receptor for factor VIIa and thus plays a central role as the initiator of the extrinsic coagulation pathway. We previously demonstrated that in postmenopausal women, oral CEE reduced PAI-1 levels. The degree of decrease in PAI-1 antigens was inversely correlated with the degree of increase in d-dimer levels, a product of cross-linked fibrin degradation by plasmin, thus providing evidence of enhanced fibrinolysis. We observed that both therapies significantly decreased tissue factor antigen and increased tissue factor activity levels from baseline values, suggesting the activation of coagulation pathways. Both therapies lowered plasma PAI-1 levels significantly, however, by 26% and 22%, respectively, from baseline values, similar to our previous studies showing 18% and 25% reduction with CEE alone, confirming enhancement of fibrinolysis. We determined no association between percent changes in tissue factor antigen or activity and percent changes in PAI-1 antigen.

In the Heart and Estrogen/Progestin Replacement Study (HERS), 2763 women with coronary artery disease were randomized to CEE 0.625 mg and MPA 2.5 mg daily or placebo. At an average follow-up of 4.1 years, there was no difference between groups in the primary outcome of the study (nonfatal myocardial infarction or coronary heart disease death). Of concern, women in the hormone-treated group experienced deep vein thromboses and pulmonary emboli. The relative risk of venous thromboembolism and pulmonary emboli in current users of hormone replacement therapy (36 versus 12), however, was similar (3-fold) to the previous reports in younger populations of postmenopausal women free of known coronary artery disease. Surprisingly, the risk of venous thromboembolism in postmenopausal women with coronary artery disease randomized to placebo in HERS was >10-fold higher (22 events per 10 000 woman-years) than previously reported. The recent report from the Postmenopausal Estrogen/Progestin Interventions (PEPI) trial may in part explain the higher risk of thromboembolism with placebo from HERS, which reported the increases in factor VIIIc, von Willebrand factor antigen, and fibrinogen concentration over time in the placebo group. Another consideration is that hormone replacement therapy may decrease or increase the risk of atherothrombosis depending on the presence of the factor V Leiden mutation. Advanced age, associated medical conditions (56%, 19%, and 13% were obese, diabetic, and smokers, respectively), sedentary lifestyle, and coronary artery disease, however, may have contributed to the higher risk of venous thromboembolism in HERS than previously reported. Furthermore, in contrast to the HERS experience, there were very few cardiovascular events in the 3-year PEPI trial of 875 healthy postmenopausal women, which included the same combination of CEE and MPA in one of the treatment groups as in HERS, but the PEPI participants were on average 10 years younger than participants in HERS, and venous thromboemboli were few.

Activation of coagulation, however, may not be balanced by activation of fibrinolysis in some postmenopausal women. Indeed, a recent study from HERS reported that CEE+MPA appeared to have a more favorable effect in women with high initial lipoprotein(a) levels than in women with low levels. Thus, hormone replacement therapy should not be initiated in women with coronary artery disease or the coexistence of other risk factors for hypercoagulability.

In conclusion, estrogen combined with natural or synthetic progestagen provided similar improvement in endothelium-dependent vasodilator responsiveness and effects on markers of inflammation, hemostasis, and fibrinolysis inhibition in healthy postmenopausal women. Although these effects of combination hormone therapy might be anticipated to reduce the risk of atherosclerosis, proof of cardiovascular benefit awaits completion of randomized clinical trials such as the Women’s Health Initiative.
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