Common Estrogen Receptor Polymorphism Augments Effects of Hormone Replacement Therapy on E-Selectin but Not C-Reactive Protein

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Background—The estrogen receptor-α (ER-α) IVS1-401 polymorphism identifies a group of women (≈20%) who have augmented effects of hormone replacement therapy (HRT) on levels of HDL cholesterol. This study sought to determine if this augmentation extends to HRT regulation of E-selectin and C-reactive protein (CRP) and to explore possible mechanisms by which this polymorphism might influence estrogen action.

Methods and Results—Serum levels of soluble E-selectin and CRP were measured at baseline and 1 year in 264 postmenopausal women randomized to treatment with oral conjugated equine estrogen (0.625 mg/d), estrogen plus progestin (medroxyprogesterone acetate 2.5 mg/d), or placebo. Women with the ER-α IVS1-401 C/C genotype receiving HRT had nearly a 2-fold greater reduction in E-selectin compared with C/T or T/T women (P for interaction=0.02). In contrast, there was no augmentation of the HRT-associated increase in CRP among the C/C women compared with C/T or T/T women (P for interaction=0.54). Of luciferase reporter constructs containing sequences spanning the IVS1-401 T/C polymorphism, expression of the construct containing the C allele was enhanced 10-fold, with cotransfection of a constitutively expressed B-myb vector. In contrast, B-myb resulted in only a 2.5-fold increase in expression of the T allele construct.

Conclusions—Women with the ER-α IVS1-401 C/C genotype have greater reductions in E-selectin but no further increases in CRP with HRT. The C allele produces a functional binding site for the transcription factor B-myb. The impact of this polymorphism on ER-α transcription and other estrogen-sensitive intermediate and clinical end points has not yet been established. (Circulation. 2002;105:1879-1882.)

Key Words: receptors • genetics • women • coronary disease

During the past several years, 5 randomized clinical trials have reported no effect of hormone replacement therapy (HRT) on clinical or anatomic progression of established atherosclerotic vascular disease.1-5 One possible explanation for these results is that the benefits of HRT on lipids and endothelial function were offset by previously unrecognized or underemphasized proinflammatory effects. However, among inflammation-related proteins, the effects of oral HRT are somewhat divergent, with consistent evidence of reductions in circulating levels of several soluble adhesion molecules, most notably E-selectin,6-7 and consistent evidence of increases in C-reactive protein (CRP).8-10 Ultimately, the cardiovascular effects of HRT may depend in large measure on the relative balance of these beneficial and adverse effects of HRT on inflammation. Recently, data have emerged indicating that the human estrogen receptor-α (ER-α) IVS1-401 T/C and other closely linked polymorphisms may augment estrogen action with respect to regulation of HDL cholesterol.11 The purpose of this study was to determine the extent to which this drug–gene interaction influences HRT regulation of E-selectin and CRP and to explore potential mechanisms for augmented estrogen action with this polymorphism.

Methods
We used the specimens collected from participants in the Estrogen Replacement and Atherosclerosis (ERA) trial, an angiographic end point trial of unopposed estrogen (conjugated equine estrogen 0.625 mg/d), estrogen plus progestin (medroxyprogesterone acetate [MPA] 2.5 mg/d orally), or placebo in 309 women with established coronary disease.2 Serum levels of E-selectin and CRP were available from baseline and after 1 year of follow-up in 264 women. Serum E-selectin was measured by ELISA with the use of reagents from R&D Systems.
Levels of E-Selectin and C-Reactive Protein by Treatment Arm and Estrogen Receptor-α IVS1-401 Genotype

<table>
<thead>
<tr>
<th>IVS1-401C/C (n=47)</th>
<th>IVS1-401C/T or T/T (n=217)</th>
<th>P for Interaction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-Selectin, ng/dL†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRT</td>
<td>47.4 (32.4)</td>
<td>52.8 (31.1)</td>
</tr>
<tr>
<td>Placebo</td>
<td>53.9 (23.7)</td>
<td>51.1 (28.4)</td>
</tr>
<tr>
<td>CRP, mg/dL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRT</td>
<td>0.35 (0.42)</td>
<td>0.42 (0.53)</td>
</tr>
<tr>
<td>Placebo</td>
<td>0.38 (0.53)</td>
<td>0.40 (1.09)</td>
</tr>
</tbody>
</table>

Values are expressed as median (interquartile range).
*Based on the F-test for the HRT–IVS1-401 genotype interaction term using log E-selectin or log CRP values.
†For E-selectin assays, there were 6 women with missing data (C/C, n=1; C/T or T/T, n=5).

The assay sensitivity is 0.1 ng/mL. The intra-assay coefficient of variation (CV) was 5.5% (mean, 113.3 ng/mL), and the interassay CV was 17.6% (mean, 53.8 ng/mL).

CRP was measured using ELISA and a high-sensitivity kit (American Laboratory Products Co). The assay sensitivity was 0.002 mg/dL. The intra-assay CV was 6.7% (mean, 1.37 mg/dL), and the interassay CV was 12% (mean, 2.23 mg/dL).

DNA was isolated from stored buffy coats with the use of a standard guanidine thiocyanate procedure, and genotyping for the ER-α IVS1-401T/C polymorphism was performed with polymerase chain reaction followed by digestion with the PvuII restriction enzyme and subsequent sizing of the resulting fragments on an agarose gel.

Statistics
Generalized linear models were used to describe the relationship among on-trial levels of E-selectin and CRP (after adjusting for baseline level), treatment assignment, and ER-α IVS1-401 genotypes. Levels of adhesion molecules and CRP were tested in the log scale; however, means and interquartile ranges in the Table are reported in the original scale. A nominal F-test for the treatment arm by genotype product term was used to test for evidence of a drug–gene interaction between HRT and the ER-α IVS1-401 polymorphism. Additional models adjusting for other potentially important confounders including age, race, diabetes, hypertension, body mass index, smoking, physical activity, alcohol consumption, and statin use produced similar results (data not shown). Exploratory data analysis indicated that the effect of unopposed estrogen and estrogen plus MPA were not significantly different; therefore, the 2 active treatment arms were combined. Similarly, preliminary models suggested that the data were best described using a recessive model in which the women with 2 copies of the C allele were contrasted with women carrying 1 or 2 copies of the T allele.

In Vitro Assays
To assess the possible impact of the ER-α IVS1-401 T/C polymorphism on myb-activated gene transcription, cultures of CV1 and 293T cells were cotransfected with a B-myb expression plasmid (pcDNA3-B-myb 1 to 561, a constitutively active allele of B-myb, provided by Dr Roger Watson, Imperial College School of Medicine, London) and 1 of 4 different luciferase reporter constructs derived from the plasmid 5xGal4Luc. In these reporter plasmids, the 5xGal4 response elements were replaced with a simple TATA box, TATA-Luc3 (negative control), 3 copies of the mybA binding site from mim-1 plus a TK promoter, 3A-TK-Luc3 (positive control), or a TATA box plus the 28 base pair sequences that contain the ER-α IVS1-401 T (common) or C (mutant) alleles, P2-TATA-Luc3 or P1-TATA-Luc3. CMV-β-gal was used to normalize luciferase activity for transfection efficiency.

Results
Overall, HRT lowered median E-selectin levels by 14.4% (baseline 51.5 ng/dL; follow-up 44.1 ng/mL; P=0.08). In contrast, HRT raised median CRP levels by 31% (baseline 0.40 mg/dL; follow-up 0.53 mg/dL; P=0.03). There were no significant differences between baseline and follow-up levels of E-selectin or CRP in the placebo group.

The ER-α IVS1-401 C/C genotype was present in 47 (18%) of the women studied. When the data were examined according to ER-α IVS1-401 genotype, women with the C/C genotype had significantly greater reduction in E-selectin than did women with C/T or T/T genotypes (24% versus 14%, P for interaction=0.02; Table 1 and Figure 1). However, there was no evidence of significant modification of estrogen effects on CRP based on IVS1-401 genotype (31% versus 30%, P=0.54; Table 1 and Figure 1).

A database search of transcription factor binding sites revealed that the IVS1-401 C allele produces a potential binding site for myb transcription factors (Figure 2A). Co-
transfection of CV1 cells with a luciferase reporter construct containing the ER-α IVS1–401 C allele (P1-TATA-Luc3) and a myb expression vector produced a >10-fold increase in luciferase activity compared with an only 2.5-fold increase observed in cells transfected with the IVS1–401 T allele (P2-TATA-Luc3) reporter (Figure 2B). The response with the P1-TATA-Luc3 construct is comparable to that observed from a plasmid with 3 copies of a canonical myb-response element arrayed in parallel (3A-TK-Luc3). Similar results were obtained using 293T cells (data not shown).

**Figure 2.** ER-α IVS1–401 C allele, but not the T allele, contains a functional myb binding site. **A,** The oligonucleotide sequence that contains the 5xGalLuc3 plasmid, and the degree of luciferase activation from these constructs in CV1 cells with and without co-transfection of the B-myb 1 to 561 expression plasmid. In these reporter plasmids, the 5xGalA14 response elements were replaced with a simple TATA box, TATA-Luc3 (negative control), 3 copies of the mybA binding site from mim-1, TK promoter, 3A-TK-Luc3 (positive control), or a TATA box plus the 28 base pair sequences corresponding to the consensus myb binding site, with bases in bold indicating the most highly conserved region. **B,** The construction of 4 luciferase reporter constructs from the 5xGalLuc3 plasmid, and the degree of luciferase activation from these constructs in CV1 cells with and without co-transfection of the B-myb 1 to 561 expression plasmid. In these reporter plasmids, the 5xGalA14 response elements were replaced with a simple TATA box, TATA-Luc3 (negative control), 3 copies of the mybA binding site from mim-1 plus a TK promoter, 3A-TK-Luc3 (positive control), or a TATA box plus the 28 base pair sequences that contain the ER-α IVS1–401 T (common) or C (mutant) alleles, P2-TATA-Luc3 or P1-TATA-Luc3. On the lower left, absolute luciferase activity is normalized for CMV-β-gal expression to adjust for transfection efficiency. On the lower right, luciferase activity in the co-transfected cells is expressed as a multiple of that observed in cells without the myb 1 to 561 expression vector.

**Discussion**

The nearly 2-fold greater reduction in E-selectin among ER-α IVS1–401 C/C women described here closely resembles a similar magnitude effect on HRT-associated increases in HDL described previously. However, the lack of a corresponding augmentation of HRT effect on CRP in ER-α IVS1–401 C/C women indicates that this drug–gene interaction may behave in a selective fashion, augmenting some effects of HRT but not others.

The mechanism by which this polymorphism augments HRT-associated reductions in E-selectin and HDL is not yet established. However, the C allele produces a myb binding site that, in the presence of B-myb, is capable of augmenting transcription of a downstream reporter construct 10-fold, suggesting that, in some settings, presence of this allele might amplify ER-α transcription or produce ER-α isoforms that have different properties than the full-length gene product. Because B-myb expression is itself responsive to estrogen activation, a signal-amplifying system producing augmented responses to estrogen in cell types that commonly express B-myb or related transcription factors is possible.

Despite these observations, it remains to be proved that the presence or absence of the IVS1–401 polymorphism alters the quantity or quality of ER-α transcripts or resulting protein. This likely will require experiments with a common cell type from human subjects who vary by the ER-α IVS1–401 genotype. If this can be established, extensive investigation will be warranted to determine which estrogen-sensitive tissues and pathways are affected, including additional studies of inflammation and lipid metabolism, as well as studies in other domains of relevance for estrogen action, including bone metabolism and carcinogenesis. In the meantime, the pattern of intermediate end points described here and previously, including augmented effects on HDL levels and E-selectin expression without a similar augmentation in levels of CRP, suggests a subset of women who may be more favorably affected by hormone therapy with respect to cardiovascular risk. Additional studies in larger cohorts of women are required to determine if these effects on intermediate end points translate into a real reduction in CHD events and whether such a benefit is enhanced or offset by augmented effects in other estrogen-sensitive domains, including risk for fractures, venous thromboembolic events, and breast cancer.

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**References**


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