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Differential Effects of E and Droloxifene on C-Reactive Protein and Other Markers of Inflammation in Healthy Postmenopausal Women

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Although increased levels of C-reactive protein have been linked to E therapy, the significance of this finding and whether it occurs with the selective ER modulators are unknown. Thirty-five healthy postmenopausal women were enrolled in a placebo-controlled, two-period cross-over design trial to evaluate the effects of 0.625 mg oral conjugated E and 60 mg droloxifene, a structural analog of tamoxifen, on serum levels of C-reactive protein, IL-6, and endothelial cell adhesion molecules. E treatment resulted in 65.8% higher levels of C-reactive protein ($P = 0.0002$) and 48.1% higher levels of IL-6 ($P < 0.001$), but also resulted in a 10.9% reduction in soluble E-selectin ($P = 0.002$) and borderline reductions in vascular cell adhesion molecule-1. In contrast, droloxifene had no effect on C-reactive protein and IL-6, but did produce a significant 11% reduction in E-selectin ($P < 0.00001$). However, droloxifene also resulted in an 11.6% increase in vascular cell adhesion molecule-1 ($P < 0.007$).

These data provide additional evidence of a proinflammatory effect of E that may have adverse cardiovascular consequences. However, these changes were also accompanied by a reduction in E-selectin, suggesting an antiinflammatory effect at the level of the endothelium. The net clinical impact of these changes is not yet well established. In contrast, droloxifene had little or no proinflammatory effects on C-reactive protein and IL-6 and had mixed effects on endothelial adhesion molecules. This observation provides additional rationale for continuing to evaluate the potential cardiovascular benefits of selective ER modulators. (*J Clin Endocrinol Metab* 86: 4216–4222, 2001)

THE HEART AND Estrogen/Progestin Replacement Study found no beneficial cardiovascular effect of 4.1 yr of E plus progestin therapy in women with established coronary disease (1). Within the overall null effect there was an unexpected increase in risk for cardiovascular events during the first year that offset an apparent benefit after 2 yr of treatment. Subsequently, several investigators have reported significantly higher levels of C-reactive protein (CRP) in women using oral hormone replacement therapy (2–5). Taken together, these observations suggest that the absence of a benefit in the Heart and Estrogen/Progestin Replacement Study could be due to a previously unrecognized proinflammatory effect of hormone replacement therapy, an effect that could alter the balance of risks and benefits of E therapy for heart disease prevention, especially in women with preexisting atherosclerosis.

Nevertheless, considerable uncertainty remains concerning the relationship among E action, CRP, and coronary heart disease. For example, it is unclear whether an E-associated increase in CRP reflects a true increase in the expression of proinflammatory cytokines (IL-1α and IL-6) and subsequent cytokine-mediated endothelial cell activation or whether it is simply due to a direct effect of E on hepatic synthesis of CRP. Most tissue culture data suggest that E suppresses the expression of inflammatory cytokines (6–9) and endothelial cell adhesion molecules [*e.g.* E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1)], and chemokines (*e.g.* monocyte chemoattractant protein-1) that are responsible for the attachment of white cells to the vessel wall and subsequent migration into the subintimal space. Furthermore, transdermal E, which avoids high intrahepatic concentrations of E, does not appear to raise CRP (10). Thus, oral E could simply induce hepatic synthesis of this protein during first pass metabolism without exerting a systemic proinflammatory state. Understanding whether or not E exerts a proinflammatory effect may have important implications with respect to E use for prevention of heart disease.

Even if E induces CRP synthesis without activation of inflammatory cytokines or endothelial cell adhesion molecules, it is not clear that this would be a benign effect. CRP may increase the risk for cardiovascular disease independent of other inflammatory processes (11–14). Therefore, it may also be helpful to identify other E agonists that do not increase levels of this protein. Selective ER modulators (SERMs), including tamoxifen and certain tamoxifen structural analogs, are a class of compounds with mixed E agonist/antagonist effects. Droloxifene is one such SERM that differs from tamoxifen by a single hydroxyl group. Tamoxifen lowers the risk for breast cancer (15) and is associated with fewer cardiovascular events in some (16, 17), but not all

Abbreviations: BMI, Body mass index; CRP, C-reactive protein; ICAM-1, intercellular adhesion molecule-1; SERM, selective ER modulator; VCAM-1, vascular cell adhesion molecule-1.

(18), studies. Therefore, it and other SERMs are being carefully considered as potential alternatives to conventional E replacement (19). However, whether these compounds share E's effects on CRP and possibly vascular inflammation is not yet well established.

We hypothesized that E treatment would be associated with increases in both IL-6 and CRP, whereas droloxifene would not. To test these hypotheses, we compared the effects of 6 wk of E and droloxifene on serum levels of CRP, IL-6, as well as endothelial cell adhesion molecules in a group of healthy postmenopausal women.

Experimental Subjects

Healthy postmenopausal women, aged 50 to 72 yr, were recruited by radio and newspaper advertisements in Winston-Salem, NC, and Boston, MA. Postmenopausal status was defined as cessation of menses for 1 yr or more or self-report of hysterectomy/oophorectomy and E2 concentrations below 92 pmol/liter and FSH concentrations above 40 IU/liter. Dyslipidemia or other cardiovascular disease risk factors may themselves be associated with increases in CRP that could obscure an effect of E agonists. Thus, women were excluded if their fasting low density lipoprotein cholesterol was greater than 5.2 mmol/liter or their fasting triglyceride level was greater than 10.3 mmol/liter. Women were also excluded if they were receiving medical therapy for diabetes mellitus or if their body mass index (BMI) was more than 35 or less than 18 kg/m^2. Women with a history of recurrent deep venous thrombosis or anticoagulant therapy for any thromboembolic disease within the previous 2 yr were excluded. Women taking vasodilators, including angiotensin-converting enzyme inhibitors, calcium channel blockers, nitrates, or anticonvulsants, were excluded, as were those who received any E or other hormone replacement within 3 months of the study. Women taking vasodilators were excluded to avoid obscuring the effects of the two regimens on brachial artery flow-mediated dilator responses (the subject of a separate report).

A total of 37 women (Winston-Salem, n = 25; Boston, n = 12) were recruited and gave written informed consent to participate in the study. One woman withdrew after the first treatment period because of an exacerbation of chronic hepatitis that was quiescent and not reported at baseline, and the blood samples from the end of the second treatment period for another woman were lost. Results reported here are from the 35 women with complete data for both arms of the protocol. The study was approved by each institution's institutional review board.

Materials and Methods

Participants received oral conjugated equine E (Premarin, Wyeth-Ayerst Laboratories, Inc., Radnor, PA; 0.625 mg/d) or oral droloxifene (Pfizer, Inc., Groton, CT; 60 mg/d) during two 6-wk treatment periods separated by a 4-wk washout. The sequence of drug exposure was determined by a computerized randomization scheme, the results of which were not available to the investigators until the end of the trial. Study investigators and participants were blind to the treatment assignment during the two treatment periods. Once enrolled, subjects were instructed to take three tablets and one capsule every morning. The tablets contained either droloxifene (one 40-mg and two 10-mg tablets) or placebo, and the capsule contained either conjugated equine E or placebo. Each subject received a 6-wk supply of study medications with active ingredients for one of the two regimens. After the completion of the first treatment period and the washout, each woman received a 6-wk supply of active ingredients for the other regimen. Compliance with each of the treatment regimens was ascertained by pill count at the end of each treatment period. Mean compliance by pill count was 97.8% during each treatment period. At the end of the second 6-wk treatment period, all subjects received a 12-d supply of open label medroxyprogesterone acetate (10 mg/d; Provera, Wyeth-Ayerst Laboratories, Inc.) to ensure the ablation of any endometrial hyperplasia. A follow-up phone interview 3 wk later documented the absence of any late adverse events or persistent vaginal bleeding.

Serum measurements of inflammatory markers

Fasting serum specimens for CRP, inflammatory cytokines, adhesion molecules, and the chemokine monocyte chemoattractant protein-1 were obtained at the beginning and end of each 6-wk treatment period and were stored at −80 C for later analysis. High sensitivity testing for CRP (20) was performed on a Behring BNII autoanalyzer (Dade Behring, Newark, DE) as described previously (21). Serum levels of IL-6 and IL-1β (BioSource International, Camarillo, CA) and E-selectin, ICAM-1, VCAM-1, and monocyte chemoattractant protein-1 (R&D Systems, Minneapolis, MN) were measured by ELISA according to the manufacturers' specifications. The lower limits of detection and coefficients of variation (in *parentheses*) for these assays are: for CRP, 6.53 nmol/liter (5.6%); for IL-6, 7.87 fmol/liter (12.6%); for IL-1β, 4.70 fmol/liter (18.2%); for E-selectin, 34.0 pmol/liter (17.6%); for ICAM-1, 3.89 pmol/liter (10.2%); for VCAM-1, 20.0 pmol/liter (10.7%); and for monocyte chemoattractant protein-1, 2300 fmol/liter (19.9%).

Statistical analysis

Data are expressed as the mean ± SD and as medians. Log transformation of CRP levels did not materially alter the inferences from the data. Therefore, all analyses and results for CRP are presented in their original scale. Repeated measures ANOVA models found no evidence of a period or order effect for baseline or a change in any variable reported here. Thus, simple paired *t* tests for means and the Wilcoxon matched pairs tests were used to compare baseline *vs.* follow-up values for each treatment. Repeated measures ANOVA was used to compare the effects of droloxifene with those of E within the same subjects. Binomial proportions were used to assess the probability of both CRP and IL-6 increasing in 35 women, assuming the underlying probability for any individual woman being 0.25.

Results

Table 1 shows the baseline characteristics of the study population. Table 2 and Figs. 1 and 2 show the effects of E and droloxifene on levels of CRP. E resulted in a 65.8% increase in mean CRP ($P < 0.0001$) compared with a nonsignificant 14% reduction with droloxifene ($P = 0.29$). C-Reactive protein increased during E therapy in 31 of 35 women (89%), but during droloxifene therapy it increased in only 13 of the same 35 women (37%). Similarly, E raised circulating IL-6 levels by 48.2% ($P < 0.0001$) compared with a nonsignificant 33.1% increase ($P = 0.07$) associated with droloxifene treatment in the same women. In the repeated measures ANOVA models, the differences between E and droloxifene in effects on both CRP and IL-6 were highly statistically significant ($P < 0.001$ for each).

Figure 2 shows changes in mean CRP *vs.* IL-6 for both treatments. In the case of E, the preponderance of points (n = 26) are in the upper righthand quadrant, whereas in the

TABLE 1. Demographic characteristics of the cohort (n = 35) at baseline

Characteristic	Mean (±SD) at baseline
Age (yr)	58.0 ± 4.8
Ht (cm)	164.7 ± 6.0
Wt (kg)	75.0 ± 15.2
BMI (kg/m^2)	26.3 ± 4.2
Waist hip ratio	0.80 ± 0.05
E2 (pmol/liter)[a]	78.8 ± 16.0
FSH (IU/liter)	73.6 ± 25.4
Time since menopause (yr)	12.5 ± 7.2

[a] The lower limit of detection for this assay is 69.75 pmol/liter; 25 of the 35 women had estradiol concentrations below the detection limit, but the level was set at 69.75 pmol/liter for the purpose of this table.

TABLE 2. Serum levels of inflammation proteins before and after 6 weeks of estrogen and droloxifene in 35 healthy postmenopausal women

	E						Droloxifene					
	Baseline		Follow-up		P^a	P^b	Baseline		Follow-up		P^a	P^b
	Mean (SD)	Median	Mean (SD)	Median			Mean (SD)	Median	Mean (SD)	Median		
CRP (nmol/L)	130.5 (130.5)	73.95	217.5 (169.65)	174.0	0.0002	<0.0001	113.1 (100.1)	82.65	95.7 (108.75)	60.9	0.29	0.12
IL-6 (fmol/L)	33.95 (33.95)	24.6	50.18 (37.39)	44.28	<0.0001	<0.0001	34.93 (25.58)	34.4	46.74 (49.2)	29.52	0.07	0.20
E-selectin	756.5 (311.1)	715.7	673.2 (331.5)	608.6	0.002	0.0002	748.0 (304.3)	773.5	664.7 (311.1)	637.5	<0.0001	<0.0001
ICAM-1	2488.6 (511.7)	2419.8	2489.7 (445.1)	2430.9	0.99	0.79	2493.1 (502.8)	2405.4	2444.2 (598.3)	2254.4	0.35	0.49
VCAM-1	4721 (1457)	4463	4436 (1131)	4229	0.11	0.08	4545 (1378)	4356	5061 (1749)	4835	0.007	0.004

CRP, C-Reactive protein; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1. Units of measure are picomoles per liter unless otherwise noted.

[a] Based on paired *t* test.

[b] Based on Wilcoxon matched pairs test.

corresponding plot for droloxifene, the points are nearly uniformly scattered around the origin. The probability that 26 of the 35 women would have elevated levels of both IL-6 and CRP after E treatment due to chance alone is less than 0.0001. The probability that the observed pattern during droloxifene therapy was due to chance is 0.64.

Figure 3 shows the effects of E and droloxifene on IL-6 and CRP after stratifying by BMI ($\leq$27.5 *vs.* $>$ 27.5 kg/m^2). At baseline, IL-6 levels were only 15% higher among women with a BMI greater than 27.5 kg/m^2 than in those with a BMI of 27.5 kg/m^2 or less ($P = 0.61$). However, CRP levels were more than 3-fold higher among the more obese women compared with the less obese women ($P < 0.001$). There was a significant interaction between E treatment and obesity for IL-6, such that women with a BMI more than 27.5 kg/m^2 had greater increases in IL-6 than women with a BMI of 27.5 kg/m^2 or less ($P = 0.005$). There were no interactions between droloxifene treatment and obesity with respect to IL-6 or CRP levels.

Both E and droloxifene lowered circulating levels of E-selectin by approximately 10% (E, $P = 0.002$; droloxifene, $P < 0.0001$; Fig. 1), but these effects were not significantly different ($P = 0.44$). In contrast, neither compound altered levels of ICAM-1, whereas the point estimates for changes in VCAM-1 were in divergent directions (E, 6% decrease, $P = 0.11$; droloxifene, 11.4% increase, $P = 0.006$; difference between E and droloxifene, $P < 0.001$). There were no changes in levels of IL-1β or monocyte chemoattractant protein-1 associated with treatment with either compound (data not shown). However, the within-subject variability of these assays would preclude detecting with confidence all but the most dramatic of differences.

Discussion

There are three principal findings from this study. First, the E-associated increases in CRP were accompanied by concomitant increases in serum levels of IL-6. Second, we were unable to detect a similar effect of droloxifene on either IL-6 or CRP levels. Finally, both E and droloxifene lowered levels of soluble E-selectin. These data suggest a complex pattern of effects of these two E agonists on markers of vascular inflammation and endothelial cell activation. If increased levels of IL-6 and CRP are indeed markers of a proinflammatory state (see below), these data support the hypothesis attributing the early increased risk in the Heart and Estrogen/Progestin Replacement Study to a proinflammatory effect of E.

E replacement therapy and acute phase reactants

In addition to the current report, two large observational studies (3, 4) and two clinical trials (2, 5) have documented significant elevations in CRP in women taking hormone replacement therapy (48–260% higher than in nonusers), and changes appeared after as little as 4 wk of treatment (5). CRP is an acute phase reactant whose hepatic production is primarily regulated by the proinflammatory cytokine IL-6 (22). Elevated levels of CRP are prospectively associated with cardiovascular events in healthy subjects (23–27) and in subjects with established vascular disease (24, 26, 28, 29). These

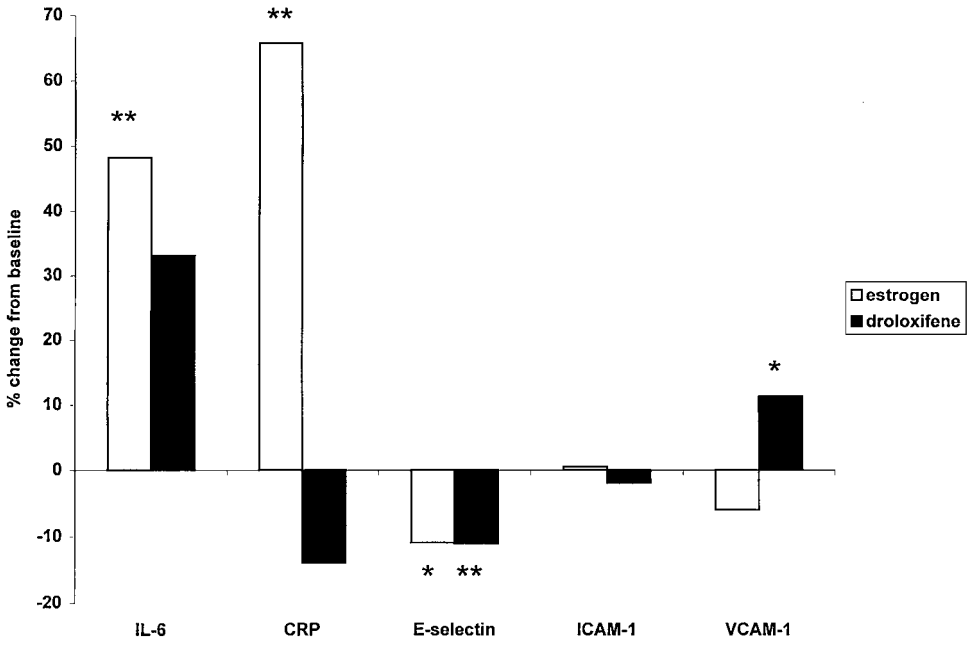

FIG. 1. Effects of E (□) and droloxifene (■) on the levels of IL-6, CRP, and soluble adhesion molecules during 6-wk regimens of E and droloxifene in 35 healthy postmenopausal women. *, $P \leq 0.01$; **, $P \leq 0.001$.

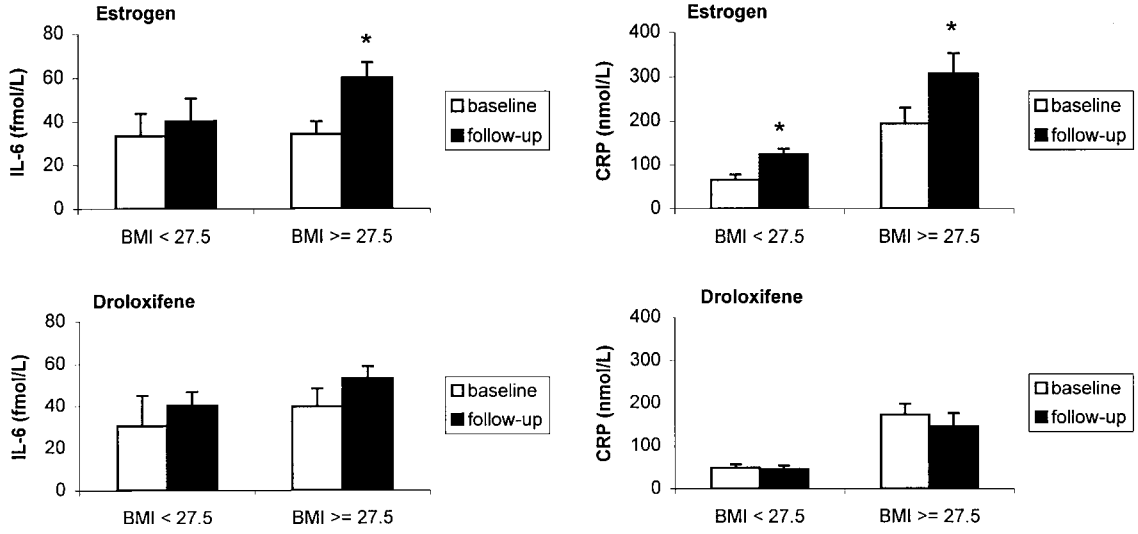

FIG. 2. Change in CRP *vs.* change in IL-6 during 6-wk regimens of E (*left panel*) and droloxifene (*right panel*) in 35 healthy postmenopausal women.

data are supported by extensive *in vitro* evidence that CRP and other inflammatory markers play an integral role in atherogenesis (30). These recent observations raise a question. Does E induce a proinflammatory state that could, at least initially, offset other favorable effects of E on risk for cardiovascular disease?

However, data concerning E's effects on CRP and other acute phase reactants are not entirely consistent. A small trial of women taking micronized 17β-E2 and 5–10 mg cyclic dydrogesterone found no effect on CRP (31). Furthermore, numerous studies have reported lower levels of other acute phase reactants in women receiving hormone replacement therapy, including fibrinogen (5, 32–34) and α_1-acid glycoprotein (4). A study in diabetic women found that transdermal E was associated with lower levels of CRP (10), raising the possibility that E-associated increases in that protein are due to a direct effect on its hepatic synthesis during first pass metabolism of oral E and not to an effect on systemic inflammation.

E and inflammatory cytokines

Numerous *in vitro* studies have demonstrated that E suppresses IL-1, IL-1 receptor, IL-6, and TNFα expression in tissue cultures of human or murine osteoblasts (6, 35, 36), marrow stromal cells (35, 37, 38), circulating monocytes (7), human umbilical vein endothelial cells (8), splenic macrophages (39), and even atherosclerotic aorta (9). On the other hand, Maret *et al.* (40) reported no effect of E on vascular smooth muscle cell production of IL-6, and Zuckerman *et al.* (41) found E-induced increases in peritoneal macrophage production of IL-6 and TNFα in culture. Unfortunately, little

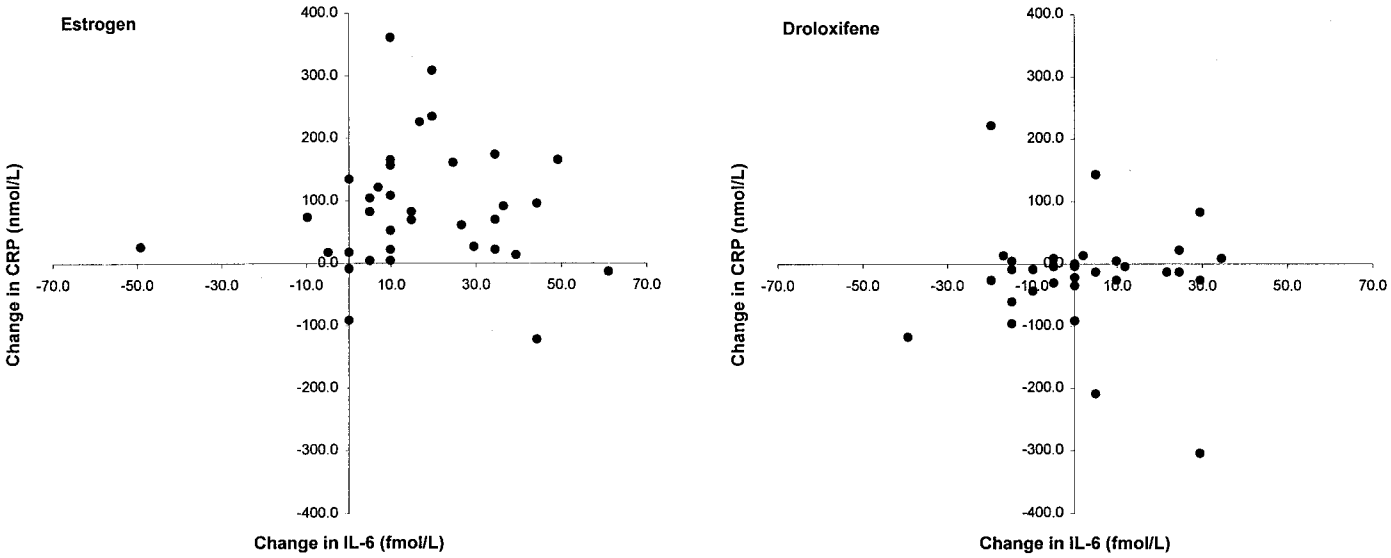

FIG. 3. Baseline (□) and follow-up (■) levels of IL-6 and CRP before and after 6 wk of treatment with E or droloxifene, stratified by BMI of 27.5 or less or 27.5 kg/m^2 or more. $P \leq 0.001$.

is known about the effects of E on circulating levels of proinflammatory cytokines in women. Cantatore *et al.* (42) reported that the loss of endogenous E after oophorectomy resulted in higher levels of IL-1 and IL-6, and that postoperative hormone replacement therapy returned them to low premenopausal levels. In contrast, in the current study we observed E-associated increases in serum levels of IL-6. The combination of significant increases in IL-6 and CRP gives greater credence to the concept that E may produce a low grade systemic proinflammatory state with potentially deleterious cardiovascular consequences (43). After treatment with E, both IL-6 and CRP levels were highest among women with elevated BMIs. More information is needed about the relation among E, obesity, and risk for clinical cardiovascular events.

E and circulating adhesion molecules

Despite the IL-6 and CRP data suggesting a proinflammatory effect of E, the adhesion molecule data are consistent with an antiinflammatory effect at the level of the endothelium. Endothelial cell adhesion molecules are induced by inflammatory cytokines and facilitate leukocyte attachment to and migration across endothelial cells during vascular inflammation. Several *in vitro* studies have shown that E can inhibit the induction of E-selectin, VCAM-1, and ICAM-1 in ER-positive endothelial cell lines (44, 45), although other *in vitro* studies have failed to observe such an effect (46, 47). Studies have also documented that E can inhibit vascular cell expression of monocyte chemoattractant protein-1, a chemokine thought to play a major role in stimulating the migration of blood monocytes into developing atherosclerotic lesions (48). More recently, this and other clinical studies have documented lower serum levels of soluble fragments of E-selectin, VCAM-1, or ICAM-1 (2, 5, 49) in women receiving E replacement. In observational studies, elevated levels of E-selectin, VCAM-1, or ICAM-1 have been associated with coronary heart disease (26, 50–52), angiographically defined coronary stenoses (53, 54), carotid atherosclerosis (53), and cardiovascular complications of type 2 diabetes (55). In addition, elevated levels of both ICAM-1 and E-selectin are associated with risk for future cardiovascular events (26, 56). Thus, E-associated reductions in circulating adhesion molecules may reflect antiinflammatory action at the endothelial level contributing to a cardioprotective effect of E. Possibly, a systemic proinflammatory effect of E is overcome at the endothelial level by E's well documented ability to upregulate endothelial cell production of nitric oxide (57), a molecule that inhibits the expression of endothelial cell adhesion molecules.

SERMs and inflammation

There are relatively fewer data on the effects of SERMs on markers of inflammation. The CRP data from this trial concerning droloxifene are consistent with previous studies of raloxifene (58, 59) that found no effect on CRP in postmenopausal women. Interestingly, in both the study by Blum *et al.* (59) and in the current study, treatment with a SERM resulted in a nonsignificant trend toward higher levels of IL-6. However, CRP levels decreased in both studies, suggesting an uncoupling of the relation between IL-6 and CRP with SERM therapy. Similar to the current study, Blum *et al.* (59) observed a significant reduction in E-selectin with raloxifene treatment. They also observed a reduction in ICAM-1 levels, an effect not seen in the current study of droloxifene. In the aggregate, these data suggest that these two SERMs, raloxifene and droloxifene, may share with E a favorable effect on endothelial activation without the E-associated increases in IL-6 and CRP.

Limitations

A randomized, double blind, cross-over study is an efficient way to observe and compare the effects of multiple therapies in the same subjects. However, this study's size

means that inferences concerning subtle effects within or between the two regimens are less certain. Furthermore, based on this short-term study, we do not know whether droloxifene's effects would persist or change over time. If other studies of E and SERMs are any indication, it is plausible that droloxifene's E agonist effects will persist with continued therapy. Finally, the degree to which modest changes in circulating levels of cytokines or adhesion molecules reflect biological activity at the vessel wall or within immune/inflammatory tissues is unclear. In particular, the links among IL-6, E-selectin, and clinical cardiovascular events are not as well established as is the relationship between CRP and cardiovascular disease. Even for CRP, elevated levels due to an effect of E or other drugs may not have the same implications as levels that occur in the absence of any drug effect. More prospective data are required to understand the pathophysiological and clinical implications of E-associated changes in inflammation markers.

Summary

In summary, in addition to raising CRP, oral E raises circulating levels of IL-6. Despite these proinflammatory effects, E lowers circulating levels of E-selectin. The clinical consequences of this mixed profile of both pro- and antiinflammatory effects of E remain unknown. Droloxifene shares with E an ability to lower levels of E-selectin, but does not raise CRP or IL-6 levels in healthy postmenopausal women. Whether the absence of an adverse effect on those molecules will make droloxifene, or similar SERMs, a more efficacious or safer form of hormone replacement needs to be established in clinical trials.

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