Effect of Lower Dosage of Oral Conjugated Equine Estrogen on Inflammatory Markers and Endothelial Function in Healthy Postmenopausal Women

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Objective—Although oral estrogen replacement therapy (ERT) in postmenopausal women improves endothelial function, it also increases plasma C-reactive protein (CRP) and interleukin-6 (IL-6) concentration. The proinflammatory effect of oral ERT may explain the increased risk of coronary heart disease (CHD) associated with this treatment. Recent observational studies have demonstrated that a lower dose of oral estrogen reduces the risk for CHD. The purpose of the present study was to investigate the effects of low-dose oral estrogen on vascular inflammatory markers and endothelium-dependent vasodilation in postmenopausal women.

Methods and Results—Postmenopausal women were randomized into 3 groups to receive no treatment (n=14) or oral conjugated equine estrogen (CEE) at a dosage of 0.625 mg (n=15) or 0.3125 mg (n=15) daily for 3 months. CEE at a dosage of 0.625 mg resulted in significant increases in plasma concentrations of CRP from 690.9 ± 749.5 to 1541.9 ± 1608.0 ng/mL, serum amyloid A from 6.12 ± 4.15 to 8.25 ± 4.40 μg/mL, and IL-6 from 1.45 ± 0.73 to 2.35 ± 1.16 pg/mL. In contrast, CEE at a dosage of 0.3125 mg had no effect on these inflammatory markers. Both dosages of estrogen significantly decreased E-selectin concentration, whereas the concentrations of intercellular and vascular cell adhesion molecules remained unchanged. In both CEE groups, flow-mediated vasodilation in the brachial artery was increased significantly, whereas nitroglycerine-induced vasodilation was unaltered.

Conclusions—Oral CEE at a low dose of 0.3125 mg in postmenopausal women eliminated the adverse effects of high-dosage oral CEE on vascular inflammatory markers in addition to preserving the favorable effects of estrogen on cell adhesion molecules and endothelial function. (*Arterioscler Thromb Vasc Biol.* 2004;24:571-576.)

Key Words: estrogen ■ postmenopausal women ■ C-reactive protein ■ endothelium ■ cell adhesion molecules

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The Heart and Estrogen/Progestin Replacement Study (HERS)[1] and the Estrogen Replacement and Atherosclerosis (ERA) Trial[2] showed that oral hormone replacement therapy (HRT) did not reduce the risk of coronary heart disease (CHD) in postmenopausal women with established coronary disease. In addition, the Women's Health Initiative (WHI) in healthy postmenopausal women without CHD demonstrated that HRT was associated with an initial increased risk of cardiovascular disease.[3] Similarly, the Women's Estrogen for Stroke Trial (WEST) found that estrogen increased the risk for either fatal stroke or more severe neurological impairment after stroke.[4]

We have shown previously that postmenopausal estrogen replacement therapy (ERT) may reduce the risk of atherosclerosis by the combined effects of reducing plasma concentrations of low-density lipoprotein (LDL), increasing high-density lipoprotein (HDL),[5] and improving endothelium-dependent vasodilation.[6] In contrast, it has been reported that oral ERT increases plasma C-reactive protein (CRP) concen-

tration.[7,8] This protein is a circulating marker of inflammation with raised plasma concentrations being an independent risk factor for cardiovascular disease in healthy postmenopausal women.[9] Myocardial events and ischemic stroke can also be predicted by an elevation in CRP.[10] In addition, serum amyloid A protein (SAA) is also elevated in patients with unstable angina.[11] Estrogen-induced increase in CRP may be independent of changes in interleukin-6 (IL-6), which in turn stimulates hepatic secretion of CRP.[12] In contrast, a prospective randomized study demonstrated that HRT increases plasma levels of inflammatory cytokines such as IL-6.[13] Therefore, it is likely that raised levels of CRP induced by oral ERT may account, in part, for the increased number of early cardiovascular events observed in these trials.

According to Grodstein et al,[14] oral conjugated equine estrogen (CEE) at a daily dosage of 0.625 mg or greater increases the risk of stroke, whereas 0.3 mg of oral CEE daily reduces this risk. Similarly, a recent study found that low-dosage, but not medium- or high-dosage, ERT decreased the

risk of myocardial infarction in diabetic women without a history of myocardial infarction.[15] These findings suggest that higher dosage oral estrogen may be atherogenic, whereas lower doses may prevent the development of atherosclerosis.

In the present study, we hypothesized that lower dosage oral estrogen would not cause the adverse effects on vascular inflammatory markers seen with high-dosage therapy. To investigate this possibility, we measured the plasma concentration of CRP, IL-6, and cell adhesion molecule, in addition to evaluating endothelium-dependent vascular reactivity in postmenopausal women receiving either standard or lower-dosage oral estrogen.

Methods

Subjects
The study subjects were 45 Japanese women who had undergone natural menopause. For inclusion in the study the women must not have had an ovariectomy or have menstruated for at least 1 year. None of the subjects was using ERT before this study or had a history of hypertension, thyroid disease, liver disease, diabetes mellitus, cardiovascular disease, and uterine or breast cancer. In addition, no subjects smoked, used caffeine or alcohol, or were currently taking any medication known to influence lipoprotein metabolism. The study design was approved by the ethics committee of Kochi Medical School, with written informed consent being obtained from each subject before admission to the study.

Study Design
The 45 subjects were assigned randomly in an open, parallel-group fashion to the ERT groups or to a control group. This was achieved by the patients opening sealed envelopes containing the group assignments, as determined by a random number generator. The subject, physician, nor investigator knew in advance whether assignment would be to the ERT or the control group. Subjects in the standard dosage estrogen group (n=15), received 0.625 mg of oral CEE, whereas those in the low-dosage estrogen group received 0.3125 mg of oral CEE (n=15) daily for 3 months. Subjects in the control group (n=14) did not receive any treatment for 3 months. One subject in the control group withdrew during the study period. Endometrial biopsy and blood samples were obtained from each subject before and after treatment.

Laboratory Assays
Plasma concentrations of total and LDL cholesterol and triglyceride were measured by the enzymatic methods previously described.[16] The concentration of HDL cholesterol was determined by similar methods after apolipoprotein B-containing lipoproteins had been precipitated with sodium phosphotungstate in the presence of magnesium chloride.[17]

The concentration of high sensitive CRP was analyzed by the Behring Latex-Enhanced CRP assay using a Behring Nephelometer Analyzer System (Dade Behring, Tokyo, Japan). SAA concentration was determined by a latex agglutination turbidimetric immunoassay (Eiken Kagaku, Tokyo, Japan). Assays for vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, E-selectin were carried out using ELISA kits (R & D Systems, Minneapolis, Minn). Plasma IL-6 concentration was measured by a chemiluminescent enzyme immunoassay (Fuji Rebio, Tokyo Japan), whereas the concentrations of plasma estrone (E1) and estradiol (E2) were determined by radioimmunoassay.

The within-assay and between-assay coefficient of variation were <4% for lipids, <8% for E1 and E2, <3% for CRP, <4% for SAA, <7% for IL-6, <10% for ICAM-1, VCAM-1, and E-selectin.

Endothelial Function
Subjects were studied between 9:00 and 11:00 AM after a 12-hour fast. After the subjects had rested in the supine position for 10

minutes, high-resolution Doppler ultrasonography equipment with a 10-MHz transducer was used to image the right brachial artery, and measure vasodilatory responses. A non-tortuous segment of the brachial artery was scanned longitudinally 4 to 5 cm above the elbow, where the clearest image could be obtained. When an adequate transducer position was located, the skin was marked and the arm kept in the same position throughout the study. After baseline images of the brachial artery were obtained and arterial flow velocity determined, a blood pressure cuff encircling the proximal portion of the arm was inflated to 250 mm Hg for 5 minutes, followed by sudden deflation. Flow velocity in the brachial artery was determined immediately and 1 minute after cuff deflation. After 10 minutes of rest, a new baseline image was obtained, followed by sublingual administration of 0.3 mg of nitroglycerin and the brachial artery imaged for the next 4 minutes. All scans were recorded on VHS videotape for later analysis with blood pressure and heart rate also being recorded during each stage of the investigation. The diameter of the brachial artery was measured from the anterior to posterior interface between the media and adventitia ("m" line) at the end of diastole, defined by the R wave on a continuously recorded ECG. Vessel diameter was measured over 4 cardiac cycles and the measurements averaged, without previous knowledge of the subject's treatment group. Flow-mediated vasodilation (FMD) was calculated as the percentage increase in arterial diameter during hyperemia and provided an index of endothelium-dependent vasodilation.[18] Percent dilation induced by nitroglycerin was calculated in a similar manner and was used as an index of endothelium-independent vasodilation.[19] In our laboratory, the intra-observer and inter-observer variability for repeated measurements are 0.03 ± 0.02 and 0.05 ± 0.03 mm, respectively. The variability of FMD performed on 2 separate days was $2.1\% \pm 0.9\%$.

Statistical Analysis
Data are expressed as the mean±standard deviation. Differences in baseline subject characteristics, concentration of hormone and inflammatory markers, and brachial artery vasodilator responses between the 3 groups were analyzed by one-way analysis of variance. Treatment-induced changes in these parameters were analyzed by Student paired t test. A value of $P<0.05$ was considered as statistically significant.

Results

General Physiological Characteristics
No significant differences were found between the treatment groups for age (control, 52.8 ± 6.9; low-dose CEE, 54.1 ± 6.8; high-dose CEE, 53.4 ± 5.1 years), body mass index (control, 22.3 ± 2.7; low-dose CEE, 21.4 ± 3.7; high-dose CEE, 21.1 ± 2.7), blood pressure, heart rate, baseline concentration of inflammatory markers, and hormones or brachial artery vasodilator responses. Histological analysis of the endometrial biopsy specimens showed no hyperplasia before or after treatment in any subject.

Lipids, Inflammatory Markers, Hormones, and Cell Adhesion Molecules
CEE at a dosage of 0.625 mg significantly reduced plasma concentrations of total and LDL cholesterol, while significantly increasing concentrations of plasma HDL cholesterol and triglyceride. Similarly, CEE at a dosage of 0.3125 mg also significantly decreased plasma concentrations of total and LDL cholesterol, but plasma concentrations of triglyceride and HDL cholesterol did not change significantly (Table 1).

CEE at a dose of 0.625 mg significantly increased the plasma concentrations of CRP, SAA, and IL-6. In contrast,

TABLE 1. Changes in Plasma Lipids

| | Control | | CEE | | | |
| | | | 0.3125 mg | | 0.625 mg | |
	Before	After	Before	After	Before	After
Total cholesterol (mg/dL)	233.8±54.0	233.0±50.4	231.9±39.6	219.1±34.4†	241.1±46.2	218.9±29.2†
Total triglyceride (mg/dL)	101.6±47.8	109.3±81.1	92.57±60.2	108.2±76.3	103.1±50.5	121.8±48.4*
HDL cholesterol (mg/dL)	68.2±16.8	67.5±22.2	68.1±16.1	69.1±13.5	63.0±13.9	68.0±13.4*
LDL cholesterol (mg/dL)	145.3±56.9	145.7±48.2	149.6±48.5	132.3±33.1†	157.5±48.6	130.2±28.0†

HDL indicates high-density lipoprotein; LDL, low-density lipoprotein.

*$P<0.05$.

†$P<0.01$.

0.3125 mg of CEE caused no significant changes in these parameters. In both CEE groups, the concentration of E-selectin decreased significantly, whereas concentrations of VCAM-1 and ICAM-1 remained unchanged (Table 2). Plasma E1 and E2 concentrations were increased significantly in the two treatment groups but remained unchanged in the control group (Table 3).

Hemodynamic Parameters and Endothelial Function

Systolic and diastolic blood pressures, heart rate, and brachial artery diameter and blood flow did not change significantly with any treatment. The percentage increase in blood flow induced by reactive hyperemia also did not change significantly (Table 3). Both dosages of CEE significantly ($P<0.001$) increased FMD (low-dose, 3.8%±3.5% versus 8.9%±3.7%; high-dose 4.5%±1.7% versus 7.5%±3.3%) (Figure 1A). Percent dilation induced by nitroglycerin was not affected significantly by any of the three treatments (Figure 1B).

Discussion

The present study demonstrated that 0.625 mg and 0.3125 mg of CEE decreased plasma concentrations of total and LDL cholesterol, consistent with a previous report.[20] This indicates that even a smaller dosage of estrogen can stimulate hepatic LDL receptor, resulting in a reduced concentration of LDL cholesterol. CEE at a dosage of 0.625 mg, but not 0.3125 mg,

increased the plasma HDL cholesterol concentrations. Because estrogen-induced inhibition of activity of hepatic triglyceride lipase leads to the elevation of plasma concentrations of HDL,[5] lower dosages of estrogen might not affect the activity of hepatic triglyceride lipase and HDL cholesterol concentrations. Plasma triglyceride concentrations were elevated by 0.625 mg, but not by 0.3125 mg, of CEE. We previously demonstrated that estrogen-induced increase in plasma triglyceride decreases the size of LDL particles that are more susceptible to oxidation.[20] However, because CEE at a dose of 0.3125 mg does not elevate plasma triglyceride, resulting in unchanged size of LDL particles that are resistant to oxidation, the antioxidant effect of estrogen can be preserved.[20]

The present study demonstrated that CEE at a dosage of 0.625 mg increased acute inflammatory proteins such as CRP and SAA. It is not clear, however, whether oral estrogen stimulated systemic inflammatory responses or alternatively simply induced hepatic synthesis of CRP without activating inflammatory cytokines such as IL-6. IL-6 has been reported to be implicated in the process of atherosclerosis plaque formation.[21] Inflammatory stimuli induce IL-6 production, which, in turn, stimulates hepatic secretion of CRP. Accordingly, if oral ERT induces a systemic proinflammatory state, an increase in CRP caused by this therapy may be accompanied by activation of inflammatory cytokines such as IL-6. However, little is known about the effects of HRT on inflammatory cytokines in postmenopausal women. Accord-

TABLE 2. Changes in Inflammatory Markers and Cell Adhesion Molecules

| | Control | | CEE | | | |
| | | | 0.3125 mg | | 0.625 mg | |
	Before	After	Before	After	Before	After
CRP (ng/mL)	462.0±324.5	421.0±275.7	616.1±625.8	938.0±994.0	690.9±749.5	1541.9±1608.0*
SAA (μg/mL)	5.81±2.84	5.43±2.58	6.82±4.87	5.71±3.38	6.12±4.15	8.25±4.40*
IL-6 (pg/mL)	1.58±0.87	1.90±1.04	1.35±0.91	1.35±1.13	1.45±0.73	2.35±1.16†
VCAM-1 (ng/mL)	501.8±103.1	491.5±78.5	591.4±104.8	631.9±115.3	547.9±96.5	540.0±98.1
ICAM-1 (ng/mL)	190.8±47.5	194±58.5	215.6±49.1	213.3±58.1	225.7±82.5	217.3±67.8
E-selectin (ng/mL)	60.1±25.4	55.6±22.2	47.3±15.2	41.4±7.7*	54.3±39.8	49.8±35.1*

CEE indicates conjugated equine estrogen; CRP, C-reactive protein; SAA, serum amyloid protein A; IL-6, interleukin-6; VCAM, vascular cell adhesion molecule; ICAM, intercellular cell adhesion molecule.

*$P<0.05$.

†$P<0.01$ vs pretreatment.

TABLE 3. Changes in Hormones, Blood Pressure, Heart Rate, and Brachial Artery Diameter and Blood Flow

	Control		CEE 0.3125 mg		CEE 0.625 mg	
	Before	After	Before	After	Before	After
Estrone (pg/mL)	27.5±11.8	21.2±10.9	20.4±8.1	85.6±47.7*	36.6±12.4	171.4±75.4*
Estradiol (pg/mL)	14.5±10.4	9.5±11.9	11.4±2.5	57.9±22.6*	12.6±5.3	73.3±36.3*
SBP (mm Hg)	125.3±10.8	120.3±14.2	128.5±16.1	134.0±19.4	124.4±13.1	123.9±15.1
DBP (mm Hg)	73.2±10.2	73.8±11.9	71.1±11.1	73.2±11.9	70.4±11.4	70.2±12.6
Heart rate (beats/min)	63.2±11.9	60.5±10.6	66.8±13.4	65.3±12.1	61.2±5.5	64.0±9.5
Baseline diameter (mm)	3.36±0.42	3.27±0.51	3.38±0.38	3.31±0.50	3.53±0.32	3.59±0.39
Baseline flow (mL/min)	108.7±76.9	106.2±81.1	94.9±55.4	103.7±78.7	146.1±98.3	121.6±60.2
Hyperemic flow (%)	749.6±453.2	600.5±251.9	594.5±289.9	670.7±282.5	491.3±297.6	591.9±374.2

CEE indicates conjugated equine estrogen; SBP, systolic blood pressure; DBP, diastolic blood pressure.
*$P<0.001$ vs pretreatment.

ing to the WHI observational study,[22] HRT-associated increases in CRP are caused by a direct hepatic pass effect of oral estrogen and not by any effect of systemic inflammation. Evidence for this mechanism was that plasma IL-6 concentrations were similar in estrogen users and in nonuser women who were free of CHD or in whom CHD subsequently developed.[22] However, the data in this study were observational and did not evaluate changes in the parameters induced by estrogen. A prospective randomized study by Herrington et al[13] demonstrated that HRT increased plasma levels of CRP and IL-6 in obese women. In addition, Brooks-Asplund et al[23] reported that HRT increased mononuclear cell-derived tumor necrosis factor-α and IL-6 secretion. Similarly, our data demonstrated that administration of 0.625 mg of CEE resulted in a concurrent increase in IL-6 and CRP concentration in lean postmenopausal women. This indicates that high-dose oral ERT may exert a proinflammatory effect that may partially offset the favorable effects of estrogen on CHD. In addition, although CRP and SAA are markers of the acute inflammatory response, CRP may have a direct role in the pathogenesis in the development of atherosclerosis. Specially, CRP activates the complement cascade[24] and has been co-localized with complement components in atherosclerotic lesions of human coronary arteries.[25] CRP stimulates the release of inflammatory cytokines[26] and induces tissue factor expression from human monocytes.[27] In contrast, CEE at a dose of 0.3125 mg did not elevate acute inflammatory proteins or IL-6 concentration, indicating that low-dose oral CEE avoids the proinflammatory effect of oral estrogen. This neutral effect of low-dose oral estrogen on inflammatory markers may account for the safer profile and lower early CHD risk, demonstrated in the HERS and WHI investigations.

Cell adhesion molecules, expressed on the membrane of endothelial cells or leukocytes after cytokine stimulation, are shed from the surface within 24 hours.[28] This action results in a direct relationship between the level of cell adhesion molecules in the plasma and the extent of atherosclerosis and occurrence of coronary events.[29] There is evidence that the concentration of cell adhesion molecules increases after menopause, whereas ERT in postmenopausal women[30] is

associated with beneficial reductions in the levels of these molecules in the plasma. We have shown previously that the plasma concentration of E-selectin, but not ICAM-1 and VCAM-1, is decreased by administration of 0.625 mg of CEE.[31] In the present study, plasma E-selectin concentration was reduced by approximately the same extent by high-dose and low-dose CEE. This finding suggests that the favorable effect of estrogen on cell adhesion molecules is preserved at the lower dosage.

Nitric oxide, an endothelial-derived relaxing factor, is released in response to increased blood flow during reactive hyperemia. As endothelium-dependent vasodilation is suppressed by nitric oxide synthase inhibitor,[32] FMD appears to represent a vasodilation-dependent effect mediated by nitric oxide derived from the endothelium. FMD correlates with invasive testing of coronary endothelial function and determines the severity and extent of coronary atherosclerosis; therefore, it is used as a noninvasive test of endothelial function to provide valuable insights into early atherosclerosis, in addition to assessing the potential of various strategies to reverse endothelial dysfunction.[33]

Previous studies have shown that ERT increases endothelium-dependent vasodilation in either the coronary arteries of cholesterol-fed ovariectomized monkeys[34] or the brachial artery of postmenopausal women.[35] The present study confirmed these findings as both high-dose and low-dose CEE increased FMD in the brachial artery, implying an improvement in endothelium-dependent vasodilation. The response to nitroglycerin, which measures endothelium-independent vasodilation, was not affected in either treatment group. Because endothelial function in the brachial artery has been reported to be impaired in patients with coronary artery disease, it is generally accepted that measures of endothelial function in the brachial artery reflect changes in the coronary arteries.[36] Taken together, our observations suggest that low-dose CEE preserved the beneficial effect of estrogen on coronary artery endothelial function without affecting the function of the vascular smooth muscle.

While smoking and obesity are known to influence CRP levels,[37,38] none of the subjects in our study smoked, and mean body mass index did not change in any treatment group
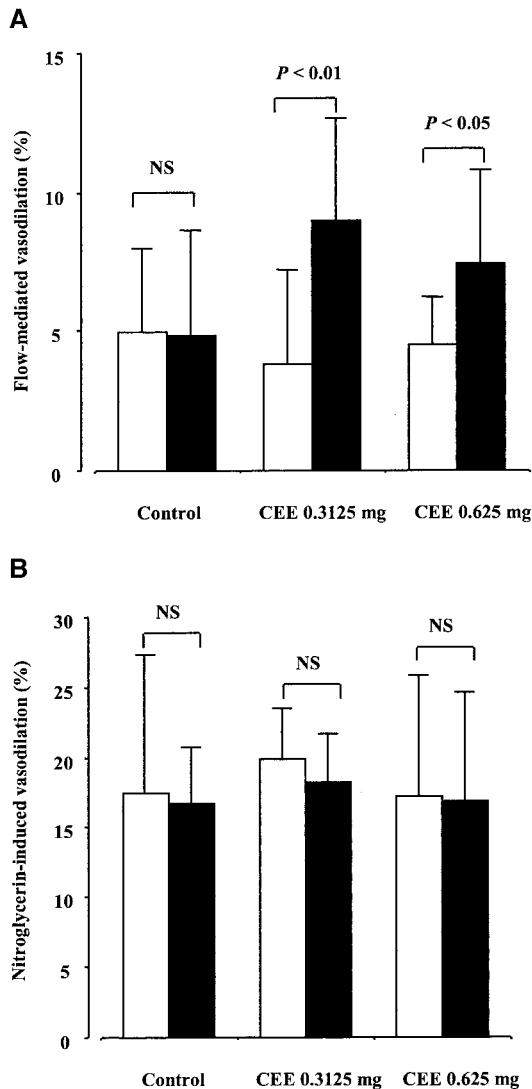
A, Effect of conjugated equine estrogen (CEE) at a dosage of 0.625 mg or 0.3125 mg on flow-mediated vasodilation in the brachial artery. NS indicates not significant. Open and closed columns indicate pretreatment and posttreatment, respectively. B, Effect of conjugated equine estrogen (CEE) at a dosage of 0.625 mg or 0.3125 mg on nitroglycerin-induced vasodilation in the brachial artery. NS indicates not significant. Open and closed columns indicate pretreatment and posttreatment, respectively.

during the study period. The study also investigated changes in the plasma concentrations of inflammatory markers and cell adhesion molecules. The clinical relevance of these parameters has been supported by several studies, although the biological function of inflammatory markers and cell adhesion molecules in sera remains unclear. In the present study, we investigated the effects of low-dose oral CEE in healthy and younger postmenopausal women. The results of the present study might be different in older postmenopausal women or women with coronary risk factors such as diabetes mellitus or hypertension. Because our subject number was relatively small and standard deviation of CRP was fairly high, additional studies are needed to determine the effects of this therapy in large patient populations.

This study demonstrated that although high-dose oral CEE exerted a proinflammatory effect by increasing acute inflammatory proteins and IL-6, lower-dose CEE was not associated with these adverse changes. In addition, low-dose oral CEE appeared to preserve the favorable effects of estrogen on cell adhesion molecules and endothelial function. The Women's Health, Osteoporosis, Progestin, Estrogen Trial has demonstrated that low-dose oral CEE also has beneficial effects on lipid metabolism[39] and vasomotor symptoms or vaginal atrophy.[40] According to other recent studies,[14,15] CEE at a daily dosage of 0.625 mg or greater increases the risk for stroke or myocardial infarction, in contrast to 0.3 mg of oral CEE daily that reduces the risk of atherosclerosis. Further studies are required to investigate whether low-dose ERT lowers CHD risk in healthy postmenopausal women and in women with established coronary disease.

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