Effect of Lower Dose of Oral Conjugated Equine Estrogen on Size and Oxidative Susceptibility of Low-Density Lipoprotein Particles in Postmenopausal Women

Akihiko Wakatsuki, MD; Yuji Okatani, MD; Nobuo Ikenoue, MD; Koichi Shinohara, MD; Kazushi Watanabe, MD; Takao Fukaya, MD

Background—Estrogen replacement therapy (ERT) has an antioxidant effect that opposes the oxidation of LDL. Oral ERT-induced increases in plasma triglyceride, however, are associated with decreased LDL size, which may counteract this antioxidant effect. Because lower doses of oral estrogen do not affect plasma triglyceride concentrations, LDL size might not change, and the antioxidant effect of estrogen might be preserved. We investigated whether a lower dose of oral estrogen could eliminate the adverse effects of high-dose oral ERT on the size and oxidative susceptibility of LDL in postmenopausal women.

Methods and Results—Postmenopausal women received no treatment or were treated with oral conjugated equine estrogen (CEE) 0.625 or 0.3125 mg/d for 3 months. CEE at a dose of 0.625 mg/d significantly increased plasma triglyceride concentrations and decreased LDL diameter, but the concentrations of LDL-derived thiobarbituric acid reactive substances (TBARS) and lag time for conjugated diene formation did not change. In contrast, 0.3125 mg of CEE did not affect plasma triglyceride concentrations or LDL diameter and significantly decreased LDL-derived TBARS concentrations and significantly prolonged LDL lag time. Estrogen-induced changes in LDL diameter correlated negatively with changes in plasma triglyceride (r = −0.44, P < 0.01) and LDL-derived TBARS (r = −0.57, P < 0.001) but positively with changes in LDL lag time (r = 0.42, P < 0.01).

Conclusions—Because oral CEE at a dose of 0.3125 mg/d 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. (Circulation. 2003;108:808-813.)

Key Words: lipoproteins ■ hormones ■ women

Smaller, denser LDL particles are associated with increased risk of coronary heart disease (CHD), in part because small LDL particles are more susceptible to oxidative modification, an initial step in the atherosclerotic process. We previously demonstrated that plasma LDL concentrations increase and the size of LDL particles decreases after menopause, whether natural or surgically induced, which suggests an increased risk for atherogenesis in women with low plasma concentrations of estrogen.

Postmenopausal estrogen replacement therapy (ERT) has beneficial effects on plasma lipids, LDL oxidation, hemostatic factors, and endothelial function. However, in postmenopausal women with established coronary disease, the Heart and Estrogen/Progestin Replacement Study (HERS) and the Estrogen Replacement and Atherosclerosis (ERA) trial showed no benefit of oral hormone replacement therapy (HRT) on the risk of CHD. In addition, the Women’s Health Initiative (WHI) in healthy postmenopausal women without CHD demonstrated an early increased risk of cardiovascular events in the HRT group.

We previously reported that oral estrogen–induced increases in plasma triglycerides were associated with reduced LDL particle size. We also demonstrated that because estrogen-induced small LDL particles are more susceptible to oxidation, the antioxidant effects of estrogen might be offset in patients with increased triglycerides. According to Ebara et al, increased oxidized LDL levels may be associated with plaque instability in human coronary atherosclerotic lesions. Therefore, an oral estrogen–induced reduction in LDL particle diameter might explain the increased number of early cardiovascular events demonstrated in the HERS and WHI trials. The Women’s Health, Osteoporosis, Progestin, Estrogen (HOPE) trial demonstrated that a lower dose of oral estrogen was not associated with the same increase in plasma triglycerides as the higher dose. Accordingly, low-dose estrogen therapy can ameliorate the adverse effects of high-dose oral ERT on the size and oxidative susceptibility of LDL.

In the present study, we hypothesized that lower-dose oral estrogen would not have the adverse effect that high-dose oral
estrogen has on the size of LDL particles. We measured plasma concentrations of lipids, the size of LDL particles, and the susceptibility of LDL to oxidative modification in postmenopausal women receiving standard or lower-dose oral estrogen.

Methods

Subjects
The study subjects were 51 naturally postmenopausal Japanese women who satisfied the following conditions during this period. These patients had not undergone ovariectomy. None of the subjects had menstruated for at least 1 year. None of them smoked, used caffeine or alcohol, or had a history of hypertension, thyroid disease, liver disease, diabetes mellitus, or cardiovascular disease. In addition, none of the women were currently taking any medication known to influence lipoprotein metabolism, and none were taking ERT before the present study. Written informed consent was obtained from each subject before admission to the study. The study design was approved by the ethics committee of Kochi Medical School.

Study Design
Fifty-one patients were randomly assigned in open, parallel-group fashion to the ERT groups or the control group. After they signed informed consent forms, the patients were randomized by opening sealed envelopes that contained the group assignments, as determined by a random number generator. Neither the subject, the physician, nor the investigator knew in advance whether assignment would be to the ERT or the control group. Subjects in the standard-dose estrogen group (n = 15) received 0.625 mg of oral conjugated equine estrogen (CEE), and those in the low-dose estrogen group received 0.3125 mg of oral CEE (n = 17) daily for 3 months. Subjects in the control group (n = 14) did not receive any treatment for 3 months. Two and three subjects withdrew during the study period from the group receiving 0.625 mg of CEE and the control group, respectively. Endometrial biopsy and blood samples were obtained from each subject before and after treatment. Venous blood samples were drawn into tubes that contained 1 mg/mL EDTA between 8 AM and 10 AM after a 12-hour fast. Samples were centrifuged immediately at 1500g for 20 minutes at 4°C to obtain plasma.

Measurement of Lipids, Hormones, and Isolation of LDL
Plasma concentrations of total cholesterol and triglyceride were measured by the enzymatic methods described previously. 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. LDL (density, 1.019 to 1.063) was fractionated from freshly drawn (<24 hours) plasma samples by ultracentrifugation according to the method of Havel et al. Concentration of cholesterol in LDL was measured by enzymatic methods. Plasma concentrations of estrone (E1) and estradiol (E2) were measured by radioimmunoassay.

LDL Particle Diameter
LDL was subjected to gradient gel electrophoresis with 2% to 15% nondenaturing polyacrylamide-agarose gels as described previously. The gels were stained with Coomassie G-250 (Nacalai, Kyoto, Japan). The distribution profile of LDL subfractions was determined by densitometric scanning of the gels at 633 nm (Shimadzu, Kyoto, Japan). The apparent diameters of major LDL subfractions were measured by comparing results with a calibration curve constructed with ferritin, thyroglobulin, and latex beads.

Susceptibility of LDL to Oxidation
To remove EDTA, the isolated LDL fraction was dialyzed for 48 hours against 30 mmol/L sodium phosphate buffer that contained 150 mmol/L NaCl. LDL 200 μg/mL was oxidized by the addition of 5 μmol/L CuSO4 and incubated at 37°C for 3 hours. The concentrations of thiobarbituric acid reactive substances (TBARS) in the LDL subfraction were determined according to the method of Ohkawa et al. In brief, 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL of a 0.8% thiobarbituric acid solution were added to the LDL solution, and the volume was brought to 4.0 mL with distilled water. The mixture was shaken thoroughly and heated in an oil bath at 95°C for 60 minutes. After the mixture was cooled with tap water, 1.0 mL of distilled water and 5.0 mL of butyl alcohol and pyridine (15:1) were added, and the sample was shaken gently for 5 minutes. After centrifugation at 1500g for 10 minutes, the butyl alcohol-pyridine phase containing the TBARS was separated, and its absorbance was measured at 532 nm. The results were expressed as moles of equivalent malondialdehyde per milligram of protein, with malondialdehyde from tetramethoxypropane used as a standard and double-distilled water used as a control.

The EDTA-free dialyzed LDL subfraction (200 μg/mL) was oxidized by addition of 2.0 μmol/L CuSO4. The kinetics of formation of conjugated diene were determined by monitoring the change in absorbance at 234 nm with a spectrophotometer equipped with a 12-position automatic sample changer (Beckman model DU 640). Absorbance was recorded at 37°C every 3 minutes for 4 hours. The lag phase, propagation phase, and decomposition phase were determined as described previously. A tangent to the curve was drawn during the propagation phase and extrapolated to the time axis. The time interval between the addition of CuSO4 and the intersection point of the tangent with the time axis was defined as lag time.

Statistical Analysis
Data are expressed as the mean ± SD. Differences between the groups in subject characteristics, baseline concentrations of hormones, diameter of LDL particles, and susceptibility of LDL to oxidation were analyzed by 1-way ANOVA. Treatment-induced changes in these parameters were analyzed by Student’s paired t test. Regression lines were determined by the least squares method. A level of P < 0.05 was accepted as statistically significant.

Results

General Physiological Characteristics
No significant differences were found between groups in age, body mass index, menopausal period, or basal concentrations of lipids (Table 1). Histological analysis of the endometrial biopsy specimens showed no hyperplasia before or after treatment.

Lipid and Hormone Concentrations
Baseline levels of lipids and hormones were not significantly different between groups. CEE at a dose of 0.625 mg

<table>
<thead>
<tr>
<th>TABLE 1. Subject Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Age, y</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
</tr>
<tr>
<td>Menopausal period, y</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; SBP, systolic blood pressure; and DBP, diastolic blood pressure.
significantly reduced plasma concentrations of total and LDL cholesterol while significantly increasing concentrations of plasma HDL cholesterol and triglyceride. Similarly, CEE at a dose of 0.3125 mg also significantly decreased plasma concentrations of total and LDL cholesterol, but plasma concentrations of triglyceride and LDL cholesterol did not change significantly. Both 0.625 and 0.3125 mg of CEE significantly increased the plasma concentrations of E1 and E2. No significant changes in lipids or hormones were observed in the control group (Table 2).

Diameter and Oxidative Susceptibility of LDL

No significant differences were found between groups in baseline LDL particle diameter and oxidative susceptibility of LDL. CEE at a dose of 0.625 mg significantly reduced the diameter of LDL particles, but 0.3125 mg of CEE did not result in significant changes. The concentration of LDL-derived TBARS and LDL lag time for conjugated diene formation after reaction with CuSO4 did not change significantly after 0.625 mg of CEE at a dose of 0.625 mg significantly reduced the diameter of LDL particles. McNamara et al21 suggested that the plasma level of triglycerides \( r = -0.59, P < 0.001 \); Figure 1A) and with the concentration of LDL-derived TBARS \( r = -0.54, P < 0.001 \); Figure 2A) but positively with LDL lag time \( r = 0.67, P < 0.001 \); Figure 3A). Estrogen-induced changes in LDL particle diameter correlated negatively with changes in plasma triglyceride concentrations \( r = -0.44, P < 0.01 \); Figure 1B) and with changes in the concentration of LDL-derived TBARS \( r = -0.57, P < 0.001 \); Figure 2B) but positively with changes in LDL lag time \( r = 0.42, P < 0.01 \); Figure 3B).

## Discussion

### Lipids and LDL Particle Size

Estrogen has been reported to decrease plasma concentrations of LDL particles by stimulating hepatic synthesis of LDL receptors.18 In the present study, both 0.625 and 0.3125 mg/d of CEE decreased plasma concentrations of total and LDL cholesterol, consistent with a previous report.11 Therefore, even a smaller dose of estrogen can stimulate the hepatic LDL receptor, resulting in a reduced concentration of LDL cholesterol. CEE at a dose of 0.625 mg but not 0.3125 mg increased the plasma HDL-cholesterol concentrations. The activity of hepatic triglyceride lipase (H-TGL) was not evaluated in the present study; however, previously reported findings demonstrate that oral ERT decreases H-TGL activity, and this estrogen-induced inhibition of H-TGL activity leads to the elevation of plasma concentrations of HDL.19 Estrogen directly enters the hepatic circulation and inhibits H-TGL activity when standard doses of estrogen are administered orally. However, because of less hepatic stimulation, lower doses of estrogen might not affect the activity of H-TGL and HDL-cholesterol concentrations.

Hypertriglyceridemia is also a risk factor for CHD.20 In the present study, 0.625 mg but not 0.3125 mg of CEE increased the plasma concentration of triglyceride and reduced the size of LDL particles. McNamara et al21 suggested that the plasma level of triglyceride is the single most important factor affecting the size of LDL particles. The present data also demonstrated that estrogen-induced changes in plasma triglycerides correlated negatively with estrogen-induced

### Table 2. Changes in Plasma Lipids and Hormone Concentrations

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.3125 mg/d</th>
<th>0.625 mg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>241.1±57.3</td>
<td>240.3±54.4</td>
<td>230.7±39.6</td>
</tr>
<tr>
<td>Total triglyceride, mg/dL</td>
<td>118.9±75.2</td>
<td>121.9±67.5</td>
<td>101.7±56.7</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>67.2±16.4</td>
<td>66.5±21.4</td>
<td>66.4±16.3</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>150.1±56.7</td>
<td>151.5±50.2</td>
<td>148.3±45.9</td>
</tr>
<tr>
<td>Estrone, pg/mL</td>
<td>27.2±11.0</td>
<td>21.2±11.0</td>
<td>31.0±9.5</td>
</tr>
<tr>
<td>Estradiol, pg/mL</td>
<td>14.2±9.7</td>
<td>9.5±11.9</td>
<td>11.2±2.4</td>
</tr>
</tbody>
</table>

\*P<0.05; †P<0.01; ‡P<0.001 vs pretreatment.

### Table 3. LDL Diameter and Susceptibility of LDL to Oxidation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>0.3125 mg/d</th>
<th>0.625 mg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>LDL diameter, nm</td>
<td>25.46±1.13</td>
<td>25.44±0.90</td>
<td>25.50±0.89</td>
</tr>
<tr>
<td>LDL-derived TBARS, nmol/200 µg</td>
<td>21.6±9.4</td>
<td>23.3±10.9</td>
<td>20.0±3.7</td>
</tr>
<tr>
<td>LDL lag time, min</td>
<td>...</td>
<td>...</td>
<td>74.8±11.6</td>
</tr>
</tbody>
</table>

P<0.05; †P<0.01 vs pretreatment.
changes in LDL particle size. These observations suggest that increases in plasma triglyceride concentration may reduce the size of LDL particles, which is consistent with our previous findings.7,8 In contrast, 0.3125 mg of CEE did not increase plasma triglyceride concentrations, and the size of LDL particles was unchanged. We previously outlined the mechanism of the estrogen-induced decrease in LDL particle size as follows. Estrogen-induced hypertriglyceridemia enhances lipid transfer reactions, which results in triglyceride-rich and cholesterol ester-poor LDL particles.22 Subsequent hydrolysis of the enriched triglyceride content by lipolytic enzymes may increase the formation of LDL particles that are smaller than normal.23

Susceptibility of LDL to Oxidation
Plasma LDL particles infiltrate the intimal space of arteries and are oxidized by oxygen free radicals. Oxidized LDL particles are readily taken up by macrophages via scavenger receptors that are not downregulated. These macrophages accumulate large amounts of cholesterol and develop into foam cells. Therefore, oxidative modification of LDL plays a key role in the development of atherosclerosis.

Biological oxidative modification can be mimicked easily by incubation in cell-free buffer containing copper ions.24 The oxidative susceptibility of LDL was evaluated by measuring the concentration of LDL-derived TBARS that resulted from lipid peroxidation of LDL and lag time for LDL, which indicates intrinsic antioxidant activity of LDL particles. In the present study, LDL-derived TBARS concentration and LDL lag time did not change after 0.625 mg of CEE, which indicates that this conventional dose of estrogen may not protect LDL against oxidative modification. Yet, some in vitro studies have shown estrogen to act as an antioxidant that inhibits the oxidation of LDL.25,26 Some clinical studies have also shown that estrogen treatment decreases the susceptibility of LDL to oxidative modification,19,27 although another study found that oxidation of LDL particles is not influenced by estrogen therapy.28 In the present study, estrogen-induced changes in LDL particle diameter correlated negatively with changes in the concentration of LDL-derived TBARS and positively with changes in LDL lag time. Although Sanchez-Quesada et al29 demonstrated that electronegative LDL does not always show evidence of increased LDL oxidative modification, the present results indicate that conventional doses of estrogen induce decreases in the size of LDL particles, which might enhance the oxidative susceptibility of LDL. We30 previously demonstrated that the size of LDL particles is reduced and LDL peroxidation is enhanced in patients whose plasma triglyceride concentrations increased at least 15 mg/dL by oral estrogen therapy. These data suggest that the antioxidative effects of estrogen are inhibited by hypertriglyceridemia-induced small LDL particles that render the particles more susceptible to oxidative modification. In contrast, our previous findings also demonstrated that the size of
LDL particles did not change and LDL peroxidation was reduced after estrogen therapy in patients whose plasma triglyceride concentrations were unchanged by oral estrogen therapy. In this group, the stable size of LDL particles preserved the antioxidative effect of estrogen. Accordingly, no overall changes in oxidative susceptibility of LDL were demonstrated in the CEE 0.625-mg group in the present study. In contrast, 0.3125 mg of CEE administration reduced LDL-derived TBARS concentrations and prolonged LDL lag time, which indicates that lower-dose ERT decreases the susceptibility of LDL to oxidative modification. Because lower-dose estrogen does not increase plasma triglyceride and produces a stable size of LDL particles, the antioxidative effect of estrogen might be preserved. Similarly, our previous findings demonstrated that transdermal ERT inhibited the oxidative susceptibility of LDL, because it decreased plasma triglyceride and produced larger LDL particles.

Study Limitations
Fasting insulin or insulin sensitivity and plasma triglyceride may be associated with the size of LDL particles. Increased insulin resistance is reportedly related to reduced LDL particle diameter. Although we did not evaluate the plasma concentrations of insulin and glucose or insulin sensitivity, subjects with diabetes mellitus were excluded in the present study. In addition, because ERT has been reported to improve insulin resistance, these factors may not adversely affect the size of LDL particles. Therefore, different doses of estrogen may affect the size of LDL particles through plasma triglyceride changes.

Conclusions
According to Grodstein et al., oral CEE at a daily dose of 0.625 mg or more increases the risk for stroke, whereas 0.3 mg of oral CEE daily is associated with a reduction in the risk for stroke. In addition, a recent study also demonstrated that low-dose but not medium- or high-dose ERT decreased the risk of myocardial infarction (MI) in diabetic women without a recent MI. These findings suggest that a higher dose of oral estrogen might increase the risk of atherosclerosis. Conversely, oral estrogen at a lower dose might preserve the favorable effects of estrogen. Similar to our findings, the HERS trial showed that oral estrogen and progestin therapy has beneficial effects on plasma concentrations of LDL cholesterol and HDL cholesterol. However, this treatment also increases plasma triglyceride concentrations. If sufficiently large, this estrogen-induced increase in plasma triglyceride can be atherogenic via a decrease in LDL particle size and can counterbalance the benefits of estrogen. In contrast, plasma triglyceride concentrations and the size of LDL particles were unaffected and the oxidative susceptibility of LDL was inhibited by lower-dose estrogen administration. Thus, low-dose ERT can ameliorate the adverse effects of oral ERT on the size and oxidative susceptibility of LDL and could have a different effect on clinical outcome. Studies are needed to investigate whether low-dose ERT is protective against the risk of CHD in healthy postmenopausal women and women with established coronary disease.

Acknowledgment
This study was supported by research grant 14571567 from the Ministry of Education of Japan.

References


Effect of Lower Dose of Oral Conjugated Equine Estrogen on Size and Oxidative Susceptibility of Low-Density Lipoprotein Particles in Postmenopausal Women
Akihiko Wakatsuki, Yuji Okatani, Nobuo Ikenoue, Koichi Shinohara, Kazushi Watanabe and Takao Fukaya

_Circulation_. 2003;108:808-813; originally published online August 4, 2003;
doi: 10.1161/01.CIR.0000084552.54277.64
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/108/7/808

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/