Different Effects of Oral Conjugated Equine Estrogen and Transdermal Estrogen Replacement Therapy on Size and Oxidative Susceptibility of Low-Density Lipoprotein Particles in Postmenopausal Women

Akihiko Wakatsuki, MD; Yuji Okatani, MD; Nobuo Ikenoue, MD; Takao Fukaya, MD

Background—Postmenopausal estrogen replacement therapy (ERT) has an antioxidant effect that opposes the oxidation of LDL particles. Oral ERT–induced increases in plasma triglyceride, however, decrease LDL particle size, which may counteract this antioxidant effect. Because transdermal ERT decreases plasma triglyceride, it may not decrease LDL particle size and may preserve estrogen’s antioxidant effect. The present study investigates whether transdermal ERT can eliminate the adverse effects of oral ERT on the size and oxidative susceptibility of LDL in postmenopausal women.

Methods and Results—Postmenopausal women received no treatment (n=12) or were treated with either 0.625 mg oral conjugated equine estrogen daily (n=16) or with transdermal estradiol (50 µg/d, n=16) for 3 months. Plasma lipids and the diameter of LDL particles were determined. Susceptibility of LDL to oxidation was analyzed by incubation with CuSO₄ and subsequent measurement of thiobarbituric acid reactive substance (TBARS) concentrations. Oral ERT significantly increased plasma triglyceride and decreased LDL diameter but did not affect LDL-derived TBARS concentrations. In contrast, transdermal ERT significantly decreased the concentrations of plasma triglyceride and LDL-derived TBARS and significantly increased LDL diameter. Estrogen-induced changes in LDL diameter correlated negatively with changes in plasma triglyceride (r=-0.51, P<0.001) and LDL-derived TBARS (r=-0.50, P<0.001).

Conclusions—Because transdermal, but not oral ERT, decreases plasma triglyceride and produces larger LDL particles that are resistant to oxidation, the antioxidant effect of estrogen can be preserved. (Circulation. 2002;106:1771-1776.)

Key Words: lipoproteins • hormones • women

Low-density lipoprotein (LDL) is heterogeneous in size and density, and not all LDL subfractions are equally atherogenic. Smaller, denser LDL particles are associated with increased risk of coronary heart disease (CHD) because they are more susceptible to oxidative modification, an initial step in the atherosclerotic process. We have previously demonstrated that plasma LDL concentrations increased and the size of LDL particles decreased after menopause, whether naturally or surgically induced. Thus, accumulated plasma LDL associated with reduced LDL particle size may be atherogenic in women with low plasma concentrations of estrogen.

Postmenopausal estrogen replacement therapy (ERT) exerts beneficial effects on plasma lipids by reducing the plasma concentrations of LDL particles and increasing those of high-density lipoprotein (HDL) particles. In addition, estrogen shows antioxidant effects by inhibiting the susceptibility of LDL and HDL to oxidative modification. 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 overall rate of coronary events and progression of coronary stenosis. In addition, the Women’s Health Initiative (WHI) in healthy postmenopausal women without CHD demonstrated that the HRT group showed an early increased risk of cardiovascular events. Similarly, the Women’s Estrogen for Stroke Trial (WEST) also demonstrated that estrogen increased the risk for fatal stroke and more severe neurological impairment after stroke. Thus, recent randomized controlled trials did not show the benefit of HRT for the risk of atherosclerosis, and trials of estrogen therapy and cardiovascular end points in healthy postmenopausal women are underway.

We previously reported that oral estrogen–induced increases in plasma triglyceride concentration can reduce the size of LDL particles. In addition, 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 showing such increased triglycer-
degradation. According to Ehara et al., increased oxidized LDL levels may be associated with plaque instability in human coronary atherosclerotic lesions. Therefore, oral estrogen–induced reduction in LDL particle diameter might explain the increased number of early cardiovascular or cerebrovascular events demonstrated in the HERS, WHI, and WEST trials.

In contrast to oral estrogen therapy, transdermal estrogen therapy has been reported to decrease plasma triglyceride concentrations. It is therefore likely that transdermal ERT may not adversely affect the size and oxidative susceptibility of LDL particles.

In the present study, to investigate whether transdermal ERT can eliminate oral estrogen’s adverse effect 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 treated with estrogen either orally or transdermally.

Methods

Subjects

The study subjects were 48 naturally postmenopausal Japanese women who satisfied the following conditions during this period: None had undergone ovariectomy; none had menstruated for ≥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; none 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 ethics committee of the Kochi Medical School approved the study.

Study Design

Forty-eight patients were randomly assigned in open, parallel-group fashion to either the ERT groups or to the control group. After signing informed consent forms, the patients were randomized by a statistician, physician, or investigator) knew in advance which patient would be assigned to the ERT or to the control group. Subjects in the oral estrogen group (n=16) received 0.625 mg conjugated equine estrogens daily, and those in the transdermal estrogen group (n=16) received 17-β-estradiol (E2) patch (absorption rate, 50 µg/d) for 3 months. Subjects in the control group (n=12: 4 subjects withdrew during the study period because of increased vasomotor symptoms) did not receive any treatment for 3 months. Endometrial biopsies and blood samples were obtained from each subject before and after treatment.

Venous blood samples were drawn into tubes containing 1 mg/mL ethylenediaminetraacetic acid (EDTA) between 8:00 am and 10:00 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 previously described. 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 the EDTA-free dialyzed LDL subfraction (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 20% acetic acid (pH 3.5) and 1.5 mL of a 0.8% TBA solution were added to the LDL solution, and the volume 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 mol equivalent malondialdehyde per mg of protein, with malondialdehyde from tetramethoxypropane used as standard and double-distilled water as a control.

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 one-way ANOVA. Treatment-induced changes in these parameters were analyzed by Student’s paired t test. Differences in the population of LDL subclass patterns were determined by McNemar’s 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, blood pressure, baseline levels of lipids and hormones, LDL particle diameter, or oxidative susceptibility of LDL (Table 1).

Lipid and Hormone Concentrations

Oral estrogen therapy significantly reduced plasma concentrations of total cholesterol, LDL cholesterol, and LDL–apolipoprotein B, while significantly increasing plasma concentrations of HDL cholesterol and triglyceride. Transdermal estrogen therapy significantly decreased plasma triglyceride concentrations but did not affect other lipid concentrations significantly. Both oral and transdermal estrogen therapies significantly increased plasma concentrations of E1 and E2, while reducing concentrations of follicle-stimulating hormone. No significant changes in lipids or hormones were observed in the control group (Table 2).

Diameter and Oxidative Susceptibility of LDL

The diameter of LDL particles was significantly reduced by oral estrogen administration and significantly increased by transdermal estrogen. Oral, but not transdermal, estrogen treatment tended to change the LDL subclass pattern from A
to B. The concentration of TBARS derived from LDL after reaction with CuSO₄ did not change significantly after oral estrogen treatment. However, transdermal estrogen therapy significantly decreased LDL-derived TBARS concentrations. No significant changes in LDL particle diameter or concentration of LDL-derived TBARS were found in the control group (Table 3). LDL particle diameter correlated negatively with the plasma level of triglyceride (before treatment, \( r = -0.76, P<0.001 \); after treatment, \( r = -0.83, P<0.001 \) (Figure 1A) and with the concentration of LDL-derived TBARS (before treatment, \( r = -0.59, P<0.001 \); after treatment, \( r = -0.70, P<0.001 \) (Figure 2A). Estrogen-induced changes in LDL particle diameter correlated negatively with changes in plasma triglyceride concentrations (\( r = -0.51, P<0.001 \)) (Figure 1B) and with changes in the concentration of LDL-derived TBARS (\( r = -0.50, P<0.001 \)) (Figure 2B).

Histological analysis of the endometrial biopsy specimens showed no hyperplasia before or after treatment.

### Table 1: Subject Characteristics

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<tr>
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<th>Oral Transdermal</th>
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<tr>
<td>Number</td>
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<tr>
<td>Age, y</td>
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<td>Menopausal period, y</td>
<td>3.6±3.0</td>
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<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>77.4±6.7</td>
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<td>Total cholesterol, mg/dL</td>
<td>234.5±56.9</td>
<td>240.3±42.6</td>
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<td>Total triglyceride, mg/dL</td>
<td>118.9±75.2</td>
<td>108.5±61.7</td>
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<td>HDL cholesterol, mg/dL</td>
<td>67.3±17.5</td>
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<td>LDL cholesterol, mg/dL</td>
<td>146.6±59.8</td>
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<td>LDL apolipoprotein B, mg/dL</td>
<td>105.5±17.2</td>
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<td>Estrone, pg/mL</td>
<td>27.4±13.8</td>
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<td>Estradiol, pg/mL</td>
<td>14.6±9.4</td>
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<td>Follicle-stimulating hormone, mIU/L</td>
<td>106.0±21.9</td>
<td>100.7±50.4</td>
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</table>

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

<table>
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<th>Control</th>
<th>Oral Transdermal</th>
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<tbody>
<tr>
<td>Total cholesterol, mg/dL</td>
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<td>240.3±54.4</td>
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<tr>
<td>Total triglyceride, mg/dL</td>
<td>118.9±75.2</td>
<td>120.6±84.7</td>
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<tr>
<td>HDL cholesterol, mg/dL</td>
<td>67.3±17.5</td>
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<tr>
<td>LDL cholesterol, mg/dL</td>
<td>146.6±59.8</td>
<td>147.6±61.7</td>
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<td>LDL apolipoprotein B, mg/dL</td>
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<td>Estrone, pg/mL</td>
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<tr>
<td>Estradiol, pg/mL</td>
<td>13.3±9.4</td>
<td>8.9±11.1</td>
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<tr>
<td>Follicle-stimulating hormone, mIU/L</td>
<td>106.0±21.9</td>
<td>96.9±35.9</td>
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</table>

\*\( P<0.01 \), †\( P<0.05 \), ‡\( P<0.001 \), vs pretreatment.

### Discussion

#### Lipids and LDL Particle Size

In the present study, oral ERT–decreased plasma concentrations of LDL cholesterol and LDL–apolipoprotein B. Each LDL particle is associated with one molecule of apolipoprotein B. Thus, the oral estrogen–induced decreases in plasma concentration of LDL–apolipoprotein B observed in the present study suggest that estrogen reduced the plasma concentration of LDL particles by removing them via LDL receptors. A decrease in the plasma level of LDL cholesterol may be accompanied by a decrease in the number of LDL particles. The plasma concentration of HDL cholesterol was increased by oral estrogen administration. 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. Thus, oral ERT has favorable effects on lipid metabolism.

In contrast, the plasma concentrations of LDL cholesterol, LDL–apolipoprotein B, and HDL cholesterol did not change significantly with transdermally administered estrogen. Because estrogen directly enters hepatic circulation when estrogen is administered orally, estrogen may stimulate hepatic LDL synthesis and inhibit H-TGL activity, which may result in favorable lipid changes. Because of less hepatic stimulation, transdermal estrogen therapy may not affect lipid metabolism.

Hypertriglyceridemia is also a risk factor for CHD. In the present study, oral estrogen therapy increased the plasma concentration of triglyceride and reduced the size of LDL particles. McNamara et al²⁰ suggested that the plasma level of triglyceride is the single most important factor affecting the size of LDL particles. Our data also demonstrated that estrogen-induced changes in plasma triglyceride correlated negatively with estrogen-induced changes in LDL particle size. These observations suggest that increases in plasma triglyceride concentrations may reduce the size of LDL particles, which is consistent with our previous findings.⁵,⁹ Accordingly, the small size of LDL particles in postmenopausal women could be decreased further by oral estrogen administration.
Two distinct LDL subclass patterns have been identified: Pattern A consists of LDL particles with a diameter of ≥25.5 nm and pattern B consists of LDL particles with a diameter of <25.5 nm. Pattern B is associated with increased plasma triglycerides, this adverse effect of estrogen is minimized by the beneficial effect of estrogen on total, LDL, and HDL cholesterol levels. In the present study, however, oral estrogen treatment changed the LDL subclass pattern from A to B in 45% of subjects. These findings suggest that oral estrogen–induced increases in plasma triglyceride may be atherogenic.

Conversely, transdermal estrogen administration decreased the plasma concentration of triglyceride and increased the size of LDL particles. Therefore, transdermal estrogen-induced reduction in plasma triglyceride may produce larger LDL particles. In addition, the prevalence of LDL subclass pattern B did not increase by transdermal estrogen administration. Lahdenpera et al. have evaluated the effects of oral (17-β E2, 2 mg, and norethisterone acetate, 1 mg per day) or transdermal (17-β E2, 0.05 mg/d, with sequential oral medroxypregesterone acetate, 10 mg/d for 12 days) estrogen therapy on plasma lipids and LDL particle size. Different from our results, they concluded that LDL particle size did not change significantly by both oral and transdermal estrogen therapies. In their study, however, oral HRT did not increase plasma triglyceride that may affect LDL particle size. Androgenic progestins such as norethisterone may decrease plasma concentrations of triglyceride and HDL cholesterol, which is consistent with their findings. In contrast, transdermal HRT decreased plasma triglyceride, consistent with the present study, but the mean LDL particle diameter seems to be greater than that in the present study. It is unlikely that transdermal HRT may increase the LDL particle size in subjects with larger LDL particles.

### 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.

Biologic oxidative modification can be mimicked easily by incubation in cell-free, buffer-containing copper ions. The oxidative susceptibility of LDL was evaluated by measuring the concentration of LDL-derived TBARS resulting from lipid peroxidation of LDL. In the present study, LDL-derived TBARS concentration did not change after oral estrogen therapy, indicating that 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. Some clinical studies have also shown that estrogen treatment decreases the susceptibility of LDL to oxidative modification, whereas another study found that the oxidation of LDL particles is not influenced by estrogen therapy. In the present study, estrogen-induced changes in LDL particle diameter correlated negatively with changes in the concentration of LDL-derived TBARS. These results indicate that oral estrogen–induced decreases in the size of LDL particles may enhance the oxidative susceptibility of LDL. We previously demonstrated that the size of LDL particles is reduced and LDL peroxidation is enhanced by oral estrogen therapy in patients whose plasma triglyceride concentrations increase. These data suggest that the antioxidative effects of

<table>
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<th>Table 3: Changes in LDL Diameter and Susceptibility of LDL to Oxidation</th>
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<td><strong>Control</strong></td>
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<tr>
<td><strong>Before</strong></td>
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<tr>
<td>LDL diameter, nm</td>
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<tr>
<td>LDL subclass pattern A/B, No. of subjects</td>
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<tr>
<td>LDL-derived TBARS, nmol/L per 200 μg</td>
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</table>

*P<0.01, †P<0.05, ‡P<0.1, vs pretreatment.

Figure 1. A, Relationship between plasma triglyceride concentration and LDL diameter. Open circles and solid line indicate pretreatment; and closed circles and dashed line, posttreatment. Pretreatment, y = −0.01x + 26.9, r = −0.76, P<0.001; posttreatment, y = −0.01x + 26.76, r = −0.83, P<0.001. B, Relationship between changes in plasma triglyceride concentration and changes in LDL diameter (y = −0.01x − 0.11, r = −0.51, P<0.001).
Estrogen Replacement and LDL Particles

estrogen can be offset 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 oral estrogen therapy in patients whose plasma triglyceride concentrations were unchanged. In this group, the stable size of LDL particles preserved the antioxidative effect of estrogen. Accordingly, oxidative susceptibility of LDL did not show overall changes with oral estrogen therapy, consistent with our previous findings. Transdermally administered estrogen, however, reduced LDL-derived TBARS concentrations. This indicates that transdermal ERT inhibits the susceptibility of LDL to oxidative modification. Because transdermal estrogen–induced decreases in plasma triglyceride may produce larger LDL particles, estrogen’s antioxidative effect might be enhanced.

Study Limitations

Fasting insulin or insulin sensitivity, as well as plasma triglyceride, may be associated with the size of LDL particles. Increased insulin resistance is reportedly related to reductions in 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.

In the present study, conjugated equine estrogen was administered orally and 17-β E2 was administered transdermally. The antioxidative effect of E2 has been reported to be greater than that of E1. According to our previous findings, however, oral conjugated equine estrogen significantly reduces LDL peroxidation in subjects without plasma triglyceride increases. This indicates that E1 as well as E2 has an antioxidative effect that may inhibit LDL peroxidation. Therefore, a different route of estrogen administration may affect the susceptibility of LDL to oxidation through LDL particle size.

Conclusions

Like our findings, the HERS trial showed that oral estrogen and progesterin therapy have 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 on plasma lipids and endothelial function. In contrast, transdermal ERT decreases plasma triglyceride concentrations and increases LDL particle size. Thus, transdermal ERT can ameliorate the adverse effects of oral ERT on size and the oxidative susceptibility of LDL and could have a different effect on clinical outcome. Studies are needed to investigate whether transdermal ERT is effective for the risk of CHD in healthy postmenopausal women and women with established coronary disease.

Acknowledgment

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References


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