Atherosclerosis

Estrogen-Induced Small Low Density Lipoprotein Particles May Be Atherogenic in Postmenopausal Women

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OBJECTIVES	The purpose of this study was to investigate the susceptibility of estrogen-induced small low density lipoprotein (LDL) particles to oxidation.
BACKGROUND	Estrogen replacement therapy in postmenopausal women has an antioxidant effect that opposes oxidation of LDL particles. Estrogen-induced increases in plasma triglyceride concentrations, however, decrease LDL particle size, which may act counter to this antioxidant effect. It has not been evaluated whether estrogen-induced small LDL particles are atherogenic.
METHODS	In 24 lean and healthy postmenopausal women treated with conjugated equine estrogen (0.625 mg daily) for three months, plasma lipid concentrations and diameter of LDL particles were measured before and after therapy. Susceptibility of LDL to oxidation was determined by measuring the concentration of thiobarbituric acid-reactive substances (TBARS) after incubation with $CuSO_4$.
RESULTS	Estrogen significantly decreased plasma concentrations of total cholesterol, LDL-cholesterol and apolipoprotein B, while increasing concentrations of triglyceride, high-density lipoprotein cholesterol and apolipoprotein A-I. Estrogen-induced changes in LDL particle diameter correlated negatively with changes in plasma triglyceride concentrations ($r = -0.55$, $p < 0.005$) and with changes in concentrations of LDL-derived TBARS ($r = -0.49$, $p < 0.005$). In subjects with substantial estrogen-induced plasma triglyceride increases, estrogen significantly reduced the diameter of LDL particles ($p < 0.05$) and significantly increased the concentration of LDL-derived TBARS ($p < 0.05$). In contrast, estrogen significantly reduced the concentration of LDL-derived TBARS ($p < 0.05$) and caused no significant change in LDL particle diameter in subjects whose plasma triglyceride concentration was unchanged with estrogen therapy.
CONCLUSIONS	Because estrogen-induced plasma triglyceride increases may produce small LDL particles that are more susceptible to oxidation, antioxidant effects of estrogen might be offset in patients showing such a triglyceride increase. (J Am Coll Cardiol 2001;37:425–30) © 2001 by the American College of Cardiology

Low density lipoprotein (LDL), a major cholesterol-carrying plasma lipoprotein, has been implicated in the formation of atherosclerotic lesions (1). Plasma concentrations of LDL-cholesterol increase after menopause (2), rendering women more susceptible to coronary heart disease (CHD) with increasing age (3). We demonstrated previously that in postmenopausal and oophorectomized women, a decrease in the plasma concentration of estrogen leads to

enhanced activity of lipoprotein lipase, which may increase the plasma LDL concentrations (4). Additionally, Arca et al. (5) have suggested that hypercholesterolemia in postmenopausal women results from impairment of the LDL receptor.

Low density lipoproteins are heterogeneous in size and density (6), and not all LDL subfractions are equally atherogenic. Smaller, denser LDL particles are associated with an increased risk of CHD (7). We previously demonstrated that the size of LDL particles decreased after menopause, whether natural or surgically induced (8). Thus, an increased plasma LDL concentration associated with a reduced size of LDL particles may be atherogenic in women with low plasma concentrations of estrogen.

Estrogen replacement therapy (ERT) in postmenopausal women exerts favorable effects on lipid metabolism by reducing the plasma concentrations of LDL particles and increasing those of high density lipoprotein (HDL) particles (9). Epidemiologic studies indicated that postmenopausal ERT significantly reduced mortality from CHD and other cardiovascular disease (10–12). In contrast, the Heart and Estrogen/progestin Replacement Study (HERS) demonstrated that estrogen and progestin therapy did not reduce the overall rate of coronary events in postmenopausal women with established coronary disease (13). We previously reported that estrogen-induced increases in plasma

Abbreviations and Acronyms

CHD	=	coronary heart disease
EDTA	=	ethylenediaminetetra-acetic acid
ERT	=	estrogen replacement therapy
E1	=	estrone
E2	=	estradiol
HDL	=	high density lipoprotein
HERS	=	Heart and Estrogen/progestin Replacement Study
LDL	=	low density lipoprotein
TBARS	=	thiobarbituric acid-reactive substances

triglyceride concentration can reduce the size of LDL particles (14,15). The resulting small LDL particles are more susceptible than larger ones to oxidative modification (16), which is considered one of the initial steps in the development of atherosclerosis. As a result, the beneficial effects of estrogen could be partially offset by a concomitant decrease in LDL particle size.

In this study, we investigated whether estrogen-induced small LDL particles are atherogenic by measuring the size of LDL particles and the susceptibility of LDL to oxidative modification in postmenopausal women treated with estrogen.

METHODS

Subjects. We studied 24 naturally postmenopausal Japanese women who satisfied the following conditions during this period: mean age, 53 years; range, 45 to 62 years; mean body mass index, 21.5 g/m^2; range, 17.7 to 27.0 g/m^2; mean menopausal period, 4.3 years; range, 1.5 to 10.0 years). These patients had not undergone ovariectomy. None of the subjects had menstruated for at least one year. None of them smoked, used caffeine or alcohol, had a history of hypertension, thyroid disease, liver disease, diabetes mellitus or cardiovascular disease, and none was currently taking any medication known to influence lipoprotein metabolism. None of the women was taking ERT before this study. No subject underwent exercise or dietary therapy during the study period. 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. Each subject received oral conjugated equine estrogen 0.625 mg daily for three months. Endometrial biopsy specimens and blood samples were obtained before and after treatment. Venous blood samples were drawn into tubes containing 1 mg/mL ethylenediaminetetra-acetic acid (EDTA) between 8:00 AM and 10:00 AM after a 12-h fast. Samples were centrifuged immediately at 1,500 × *g* for 20 min at 4°C to obtain plasma. Compliance with estrogen therapy was assessed from plasma concentrations of estrone (E1) and estradiol (E2) measured by radioimmunoassay (17) before and after therapy.

Measurement of lipids and isolation of LDL. Plasma concentrations of total cholesterol and triglyceride were measured by enzymatic methods as previously described (18). 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 (19). Plasma concentrations of apolipoprotein A-I, A-II and B were measured by a turbidimetric immunoassay (19). Low-density lipoprotein (density, 1.019 to 1.063) were fractionated from freshly drawn (<24 h) plasma samples by ultracentrifugation according to the method of Havel et al. (20). Concentrations of LDL-cholesterol were assayed enzymatically (18).

LDL particle diameter. Low density lipoprotein was subjected to gradient gel electrophoresis using 2% to 15% nondenaturing polyacrylamide-agarose gels. The apparatus was filled with a buffer consisting of 0.025 mol/L Tris, 0.192 mol/L glycine and 0.1% sodium dodecyl sulfate at a pH of 8.4. After a 2-h preelectrophoresis at 200 volts, 5 μL aliquots of the LDL fraction (5 to 10 μg protein) were applied to the gel. A reference protein mixture (Molecular Weight Marker, Daiichi, Tokyo, Japan) and carboxylated latex beads (Duke Science, Palo Alto, California) were used as standards for molecule size and mass. Electrophoresis was performed at 4°C for 26 h as follows: 2 h at 30 volts, 12 h at 50 volts and 12 h at 150 volts (21,22). The gels were then fixed in 40% acetic acid for 1 h, stained for 45 min in 0.1% Coomassie G-250 (Nacalai, Kyoto, Japan) prepared in 10% acetic acid and 30% ethanol and then destained in a mixture of 7.5% acetic acid and 10% ethanol. The gels were subjected to a gentle horizontal rotation during the fixing, staining and destaining. The distribution profile of the LDL subfractions was determined by densitometric scanning of gels at 633 nm (Shimadzu, Kyoto, Japan). The apparent diameter of the major LDL subfractions was determined by comparing the results with a calibration curve constructed using ferritin (12.20 nm), thyroglobulin (17.00 nm) and latex beads (38.00 nm) as reference samples (22).

Susceptibility of LDL to oxidation. To remove EDTA, the isolated LDL fraction was dialyzed in darkness at 4°C for 48 h against 30 mM sodium phosphate buffer containing 150 mM NaCl, which was made oxygen-free by vacuum degassing followed by purging with nitrogen. The buffer was changed after 24 h of dialysis. The EDTA-free dialyzed LDL subfraction (200 μg/mL) was oxidized by incubating at 37°C for 3 h after the addition of 5 μM $CuSO_4$. Concentrations of thiobarbituric acid-reactive substances (TBARS) in the LDL fraction were determined according to the method of Ohkawa et al. (23). In brief, 1.5 mL of 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 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 min. After the mixture was cooled with tap water, 1.0 mL of distilled water and 5.0 mL of butyl alcohol

Table 1. Changes in Plasma Lipids and Lipoproteins

	Pretreatment	Posttreatment	p Value
Total cholesterol (mg/dL)	240.24 ± 45.12	219.14 ± 30.54	0.0016
Triglyceride (mg/dL)	116.13 ± 72.09	135.25 ± 69.75	0.0107
HDL-cholesterol (mg/dL)	62.52 ± 12.38	65.0 ± 13.58	0.0399
LDL-cholesterol (mg/dL)	157.54 ± 46.14	129.59 ± 29.92	0.0001
Apolipoprotein A-I (mg/dL)	153.10 ± 20.87	162.75 ± 24.61	0.0401
Apolipoprotein A-II (mg/dL)	37.51 ± 5.29	39.43 ± 6.87	0.2183
Apolipoprotein B (mg/dL)	114.53 ± 30.47	104.97 ± 24.89	0.0028

HDL = high-density lipoprotein; LDL = low density lipoprotein.

containing pyridine (15:1, v/v) were added, and the sample was shaken gently for 5 min. After centrifugation at 1,500 × g for 10 min, the butyl alcohol-pyridine phase containing the TBARS was separated, and absorbance was measured in this phase at 532 nm. The results are expressed as molar equivalents malondialdehyde per milligrams of protein using malondialdehyde derived from tetramethoxypropane as a standard and double-distilled water as a control.

Statistical analysis. Data are expressed as the mean ± standard deviation. Changes in lipid concentrations and diameters of LDL particles were analyzed by a paired *t* test. Regression lines were determined by the least squares method. Differences in the population of LDL subclass patterns were determined by McNemar's test. A level of p < 0.05 was accepted as statistically significant.

RESULTS

Histological analysis of the endometrial biopsy specimens showed no hyperplasia before or after treatment. Estrogen treatment significantly increased the plasma concentrations of E1 (47.2 ± 30.8 pg/mL to 182.2 ± 28.5 pg/mL, p < 0.001) and those of E2 (14.9 ± 6.1 pg/mL to 56.9 ± 26.6 pg/mL, p < 0.001). Estrogen therapy significantly reduced plasma concentrations of total cholesterol, LDL-cholesterol and apolipoprotein B and significantly increased plasma concentrations of HDL-cholesterol, triglyceride and apolipoprotein A-I (Table 1). The diameter of LDL parti-

Table 2. Changes in the Concentration of LDL-derived TBARS, Diameter of LDL Particles and the Prevalence of LDL Subclass Patterns

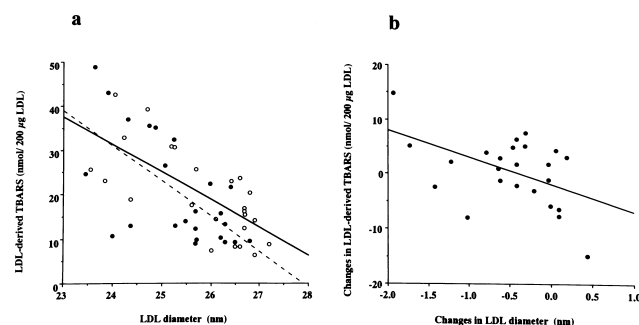|	Pretreatment	Posttreatment	p Value
LDL-derived TBARS (nM/200 μg)	20.11 ± 10.00	20.52 ± 24.89	0.7494
LDL-diameter (nm)	25.80 ± 1.12	25.32 ± 0.97	0.0008
LDL subclass pattern A/B (no. of subjects)	16/8	10/14	0.0412

LDL subclass pattern A, LDL particle diameter at least 25.5 nm; LDL subclass pattern B, LDL particle diameter less than 25.5 nm.
    LDL = low-density lipoprotein; TBARS = thiobarbituric acid-reactive substances.
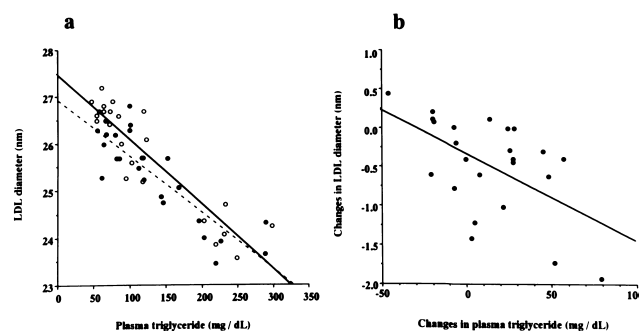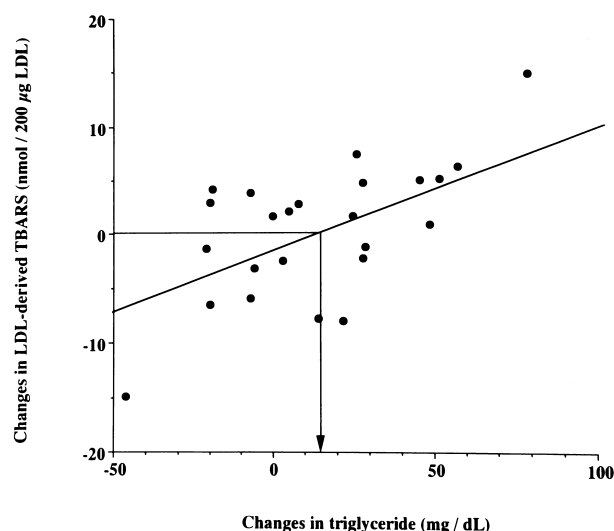

Figure 1. (A) Relationship between plasma triglyceride concentrations and LDL diameter. **Open circles** and **solid line** indicate pretreatment. **Closed circles** and **dashed line** indicate posttreatment. Pretreatment: y = −0.014x + 27.47, r = −0.91, p < 0.001; posttreatment: y = −0.012x + 26.94, r = −0.86, p < 0.001. **(B)** Relationship between estrogen-induced changes in plasma triglyceride concentrations and changes in LDL diameter (y = −0.011x − 0.33, r = −0.55, p < 0.005). LDL = low density lipoprotein.

cles was significantly reduced by estrogen treatment. Estrogen changed the LDL subclass pattern significantly from A to B in six subjects. Overall, concentrations of TBARS derived from LDL after reaction with CuSO₄ did not change significantly by estrogen treatment (Table 2). Low-density lipoprotein diameter was correlated negatively with plasma level of triglyceride (pretreatment: r = −0.91, p < 0.001; posttreatment: r = −0.86, p < 0.001) (Fig. 1A) and with the concentration of LDL-derived TBARS (pretreatment: r = −0.70, p < 0.001; posttreatment: r = −0.64, p < 0.001) (Fig. 2A). Estrogen-induced changes in LDL particle diameter showed a negative correlation with changes in the plasma triglyceride concentrations (r = −0.55, p < 0.005) (Fig. 1B) and with changes in the concentration of LDL-derived TBARS (r = −0.49, p < 0.005) (Fig. 2B). Estrogen-induced changes in plasma triglyceride concentrations showed a significant positive correlation with changes in the concentration of LDL-derived TBARS (r = 0.66, p < 0.001) (Fig. 3). According to the regression equation, the greatest estrogen-induced plasma

Figure 2. (A) Relationship between LDL diameter and the concentration of LDL-derived TBARS. **Open circles** and **solid line** indicate pretreatment. **Closed circles** and **dashed line** indicate posttreatment. Pretreatment: y = −6.22x + 180.63, r = −0.70, p < 0.001; posttreatment: y = −7.97x + 222.43, r = −0.64, p < 0.001. **(B)** Relationship between estrogen-induced changes in LDL diameter and changes in the concentration of LDL-derived TBARS (y = −4.97x − 1.99, r = −0.49, p < 0.005). LDL = low density lipoprotein; TBARS = thiobarbituric acid-reactive substances.

Figure 3. Relationship between estrogen-induced changes in plasma triglyceride concentration and changes in the concentration of LDL-derived TBARS (y = 0.11x −1.66, r = 0.66, p < 0.001). LDL = low density lipoprotein; TBARS = thiobarbituric acid-reactive substances.

triglyceride increase not associated with an increase in LDL-derived TBARS was 15 mg/dL (Fig. 3). Accordingly, subjects were divided into two groups with estrogen-induced plasma triglyceride increases either greater or less than 15 mg/dL.

In subjects whose plasma triglyceride increase was less than 15 mg/dL, estrogen significantly increased the plasma concentrations of HDL-cholesterol and apolipoprotein A-I. In contrast, concentrations of HDL-cholesterol and apolipoprotein A-I were not altered in subjects whose plasma triglyceride increased more than 15 mg/dL. Plasma concentrations of total cholesterol, LDL-cholesterol and apolipoprotein B were significantly reduced by estrogen treatment in both groups (Table 3). In subjects whose plasma triglyceride concentration increased less than 15 mg/dL, estrogen treatment significantly reduced the concentration of LDL-derived TBARS, but LDL particle diameter and prevalence of LDL subclass patterns did not change significantly. In contrast, estrogen significantly increased the concentration of LDL-derived TBARS, significantly reduced LDL particle diameter and tended to increase the prevalence of subclass pattern B among subjects whose plasma triglyceride increase was at least 15 mg/dL (Table 4).

DISCUSSION

Effects of estrogen on lipids and LDL particle size. Estrogen lowers plasma concentrations of LDL particles by stimulating hepatic synthesis of LDL receptors while increasing plasma concentrations of HDL particles via inhibition of hepatic triglyceride lipase activity (9). Consistent with these findings, this study demonstrated that the concentrations of LDL-cholesterol and of apolipoprotein B, a major apolipoprotein in LDL particles, decreased with estrogen therapy, while concentrations of HDL-cholesterol and apolipoprotein A-I, a major apolipoprotein in HDL particles, increased. Although estrogen has favorable effects on lipoprotein metabolism, it increases plasma triglyceride concentrations. Hypertriglyceridemia is also a risk factor for CHD (24). In this study, estrogen therapy increased the plasma concentration of triglyceride and reduced the size of LDL particles. Estrogen-induced changes in plasma triglyceride correlated negatively with the size of LDL particles. These observations suggest that a plasma triglyceride increase may reduce the size of LDL particles, consistent with our previous findings (14,15). Accordingly, the small size of LDL particles in women with low plasma estrogen levels could be decreased further by estrogen therapy. We previously outlined the mechanism of the estrogen-induced decrease in LDL particle size as follows: estrogen-induced hypertriglyceridemia enhances lipid transfer reactions, resulting in triglyceride-rich and cholesterol ester-poor LDL particles (25); subsequent hydrolysis of the enriched triglyceride content by lipolytic enzymes may increase the formation of LDL particles that are smaller than normal.

Two distinct LDL subclass patterns have been identified: pattern A consists of LDL particles with a diameter of at least 25.5 nm, and pattern B consists of LDL particles with a diameter of less than 25.5 nm (26). Pattern B is associated with an increased incidence of CHD. Campos et al. (27) have shown that ERT decreased the proportion of large LDL and increased the proportion of small LDL, concluding that this adverse effect of estrogen is minimized by the beneficial effect of estrogen on total, LDL and HDL-cholesterol levels. In this study, however, estrogen treatment changed the LDL subclass pattern from A to B in 25% of subjects, indicating that an estrogen-induced increase in triglyceride may be atherogenic.

Table 3. Changes in Plasma Lipids and Lipoproteins According to Triglyceride Increase

	Triglyceride Increase <15 mg/dL (n = 13)			Triglyceride Increase ≥15 mg/dL (n = 11)		
	Pretreatment	Posttreatment	p Value	Pretreatment	Posttreatment	p Value
Total cholesterol (mg/dL)	242.55 ± 49.90	217.50 ± 35.11	0.0151	237.07 ± 40.71	220.11 ± 26.96	0.0092
Triglyceride (mg/dL)	137.71 ± 86.08	131.00 ± 78.58	0.1756	99.20 ± 54.03	141.20 ± 58.70	0.0001
HDL-cholesterol (mg/dL)	65.55 ± 14.42	70.91 ± 12.46	0.0388	58.38 ± 11.02	61.02 ± 13.61	0.3685
LDL-cholesterol (mg/dL)	156.33 ± 53.52	129.03 ± 33.09	0.013	159.22 ± 37.10	130.43 ± 26.62	0.0025
Apolipoprotein A-I (mg/dL)	154.00 ± 25.91	167.36 ± 25.1	0.0484	151.86 ± 12.57	158.37 ± 25.55	0.3592
Apolipoprotein A-II (mg/dL)	35.73 ± 4.05	38.10 ± 7.00	0.4278	39.96 ± 6.06	41.83 ± 5.74	0.3734
Apolipoprotein B (mg/dL)	106.18 ± 30.32	95.55 ± 25.01	0.0256	126.01 ± 28.68	111.80 ± 22.42	0.0052

HDL = high density lipoprotein; LDL = low density lipoprotein.

Table 4. Changes in the Concentration of LDL-derived TBARS, Diameter of LDL Particles and the Prevalence of LDL Subclass Patterns According to Triglyceride Increase

	Triglyceride Increase <15 mg/dL (n = 13)			Triglyceride Increase ≥15 mg/dL (n = 11)		
	Pretreatment	Posttreatment	p Value	Pretreatment	Posttreatment	p Value
LDL-derived TBARS (nM/200 μg)	19.53 ± 9.70	16.65 ± 10.37	0.0359	21.29 ± 19.00	25.54 ± 12.32	0.0234
LDL-diameter (nm)	25.60 ± 1.27	25.27 ± 1.12	0.1172	26.04 ± 0.92	25.44 ± 0.83	0.0161
LDL-subclass pattern A/B (no. of subjects)	8/5	7/6	0.7728	8/3	3/8	0.0736

LDL subclass pattern A, LDL particle diameter at least 25.5 nm; LDL subclass pattern B, LDL particle diameter less than 25.5 nm.
LDL = low density lipoprotein; TBARS = thiobarbituric acid-reactive substances.

Effects of estrogen on 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 (28). 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 (29). In this study, LDL peroxidation induced by incubating LDL with $CuSO_4$ was measured as TBARS, which indicate the susceptibility of LDL to oxidation. Thiobarbituric acid-reactive substances did not show overall changes with estrogen therapy, which indicates that, generally, estrogen did 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 (30,31). Some clinical studies have also shown that estrogen treatment inhibited the susceptibility of LDL to oxidative modification (9,32), while another study found that oxidation of LDL particles was not influenced by estrogen (33). However, estrogen-induced changes in LDL particle diameter correlated negatively with changes in the concentration of LDL derived TBARS in this study, indicating that an estrogen-induced decrease in the size of LDL particles increases the susceptibility of LDL to oxidation. A positive correlation was observed between estrogen-induced changes in plasma triglyceride concentrations and changes in the concentration of LDL-derived TBARS. Thus, estrogen-induced hypertriglyceridemia may produce small LDL particles that show increased susceptibility to oxidative modification.

Relationship between estrogen-induced changes in plasma triglyceride and lipids, LDL particle size and LDL oxidation. The regression equation between changes in plasma triglyceride concentration and changes in the concentration of LDL-derived TBARS indicated that subjects with increased concentrations of LDL-derived TBARS had plasma triglyceride elevations greater than 15 mg/dL, and this value was used to divide subjects into two groups with greater or smaller plasma triglyceride increases. Plasma HDL-cholesterol and apolipoprotein A-I concentrations were not changed by estrogen therapy in patients whose plasma triglyceride concentrations increased beyond cutoff values, while those parameters were elevated in patients whose plasma triglyceride concentrations did not increase. The plasma HDL-cholesterol concentration has been reported to be inversely associated with plasma triglyceride concentrations; a low plasma HDL-cholesterol concentration in patients with hypertriglyceridemia could result from increased lipid transfer reactions via cholesterol ester transfer protein (34). Thus, an estrogen-induced increase in plasma triglyceride may reduce the amount of HDL-cholesterol in plasma, which was increased by estrogen.

Estrogen decreased plasma concentrations of total cholesterol, LDL-cholesterol and apolipoprotein B in both of our triglyceride-defined groups. However, the size of LDL particles was reduced, and the LDL peroxidation was enhanced by estrogen therapy in the group whose plasma triglyceride concentrations increased. Small, dense LDL particles are considered to be easily oxidized because these particles contain low concentrations of antioxidants and high concentrations of polyunsaturated fatty acids (35). Our findings indicate that antioxidative effects of estrogen can be offset by hypertriglyceridemia-induced small LDL particles that render the particles more susceptible to oxidative modification. In contrast, the size of LDL particles did not change, and LDL peroxidation was reduced after estrogen therapy in our group of patients whose plasma triglyceride concentrations were unchanged. In this group, a stable size of LDL particles preserved the antioxidative effect of estrogen.

Study limitations. We investigated lean and healthy postmenopausal women. Obese women with hypertriglyceridemia or women with CHD might respond differently. A reduction in the plasma level of triglyceride might be needed before ERT in the subjects with hypertriglyceridemia. Further studies are required to clarify these issues. Since our subject number was relatively small, the 15 mg/dL plasma triglyceride increase that served as the cutoff in this study might not apply in large patient populations.

Conclusions. According to the findings of HERS, estrogen and progestin therapy had beneficial effects on plasma concentrations of LDL-cholesterol and HDL-cholesterol, but the plasma triglyceride concentration was increased (13), as in our results. If sufficiently large, this estrogen-induced increase in plasma triglyceride can be atherogenic via a decrease in LDL particle size and partially offset the beneficial effects of estrogen. Studies are needed to investigate whether or not a reduction in plasma triglyceride concentrations during hormone replacement therapy can

reduce the risk of cardiac events in postmenopausal women with established coronary disease.

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